Role of Wnt/ β-Catenin Signaling in Neural Crest Development

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der Universität Zürich

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Zürich, 2012

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1 Summary

The neural crest is a transient population of migratory cells unique to vertebrate development. It consists of neural crest stem cell (NSCS). Stem cells are able to self-renew, meaning they can go through numerous cycles of cell division while maintaining the undifferentiated state. Additionally, they are able to give rise to multiple cell types, an ability referred to as multipotency. The population of NCSC emigrates from the dorsal neural tube shortly after neural tube closure, migrates through the body along distinct pathways, and populates different target sites where they give rise to a wide array of cell types. Derivatives of the neural crest include sensory and autonomic neurons as well as glia of the peripheral nervous system, chromaffin cells of the adrenal glands, pigment cells in the skin, or bone and cartilage of the head. Several extracellular growth factors as well as intracellular cues have been identified to influence the fate of NCSC. Among the signaling cascades playing an important role in neural crest development is the Wnt/ β-Catenin pathway.

Canonical Wnt/ β-Catenin signaling is involved in many processes throughout embryonic development and in adult tissue homeostasis. It plays multiple roles in neural crest development. First, it is necessary for induction of the neural crest at the neural plate border. Additionally, together with bone morphogenic protein (BMP) growth factors, it is able to maintain NCSC in an undifferentiated state. Furthermore, Wnt signaling is involved in generation of the sensory and melanocyte lineages. The function of Wnt/ β-Catenin signaling in sensory and melanocyte lineage formation will be the subject of this thesis.

The first publication of my thesis demonstrates a requirement for β-Catenin in specification of the sensory lineage and of melanocytes. Mouse embryos lacking β-Catenin specifically in NCSC exhibited a complete absence of dorsal root ganglia, a structure composed of sensory cells. Moreover, these mutants were deficient for melanocytes.

In the frame of the second publication Wnt/ β-Catenin was identified as an instructive signal for sensory fate acquisition. Expression of a constitutively activated form of β-Catenin in NCSC promoted sensory fate at the expense of other lineages. Furthermore, individual NCSC cultured in the presence of Wnt were instructed to adopt a sensory fate.

In the third part of my thesis I investigated the role of canonical Wnt/ β-Catenin signaling in melanocyte fate acquisition. The fact that β-Catenin is required for formation of the melanocyte lineage but NCSC in the presence of Wnt chose a sensory rather than a melanocytic fate brought up the idea of a stage- dependent response of NCSC to Wnt signaling. By expressing a stabilized form of β-Catenin at different stages of neural crest and early melanocyte development we demonstrated that β-Catenin activation can promote the generation of melanoblasts only during a narrow time window of neural crest development. These results are consistent with the hypothesis of a temporal control of neural crest lineage generation in response to canonical Wnt/ β-Catenin signaling.

2 Zusammenfassung

Die Neuralleiste ist eine vorübergehende Struktur die nur während der Embryonalentwicklung von Wirbeltieren gebildet wird und aus einer Population von migrierenden Zellen besteht. Diese Zellen werden Neuralleisten-Stammzellen genannt. Stammzellen besitzen die Fähigkeit, sich selber zu erneuern. Das bedeutet, sie können mehrere Zellteilungszyklen durchlaufen und dabei fortwährend neue Stammzellen generieren. Zudem können sie zu einer Vielzahl von verschiedenen Zelltypen differenzieren, diese Eigenschaft wird Multipotenz genannt. Die Neuralleisten-Stammzellen emigrieren vom dorsalen Neuralrohr kurz nachdem es geschlossen wurde, wandern durch den Embryo entlang festgelegten Wegen und bleiben dann an verschiedenen Zielorten wo sie zu unterschiedlichen Zelltypen differenzieren. Aus der Neuralleiste entstehen unter anderem sensorische und autonome Nerven- und Gliazellen des peripheren Nervensystems, Chromaffinzellen in den Nebennieren, Pigmentzellen in der Haut oder Knorpel und Knochen des Kopfes. Von verschiedenen extrazellulären Wachstumsfaktoren und intrazellulären Signalen wurde gezeigt, dass sie das Schicksal der Neuralleisten-Stammzellen beeinflussen. Der Wnt/ β-Catenin Signalweg ist einer davon, er spielt eine wichtige Rolle während der Entwicklung der Neuralleistenzellen.

Der kanonische Wnt/ β-Catenin Signalweg steuert viele Prozesse während der Embryonalentwicklung und im adulten Organismus. Er spielt mehrfach eine Rolle während der Neuralleistenentwicklung. Zuerst wird er für die Bildung der Neuralleiste an der Grenze zwischen Neuralrohr und umgebendem Ektoderm benötigt. Dann führen Wnt Signale dazu, zusammen mit BMP Wachstumsfaktoren, dass die Neuralleisten-Stammzellen in einem undifferenzierten Zustand bleiben. Ausserdem

spielt der Wnt Signalweg eine wichtige Rolle bei der Bildung von sensorischen Zellen und von Pigmentzellen (Melanozyten). In dieser Doktorarbeit wurde die Funktion von Wnt/ β-Catenin Signalen in der Entstehung von sensorischen Zellen und Melanozyten untersucht.

Die erste in der Doktorarbeit aufgeführte Publikation zeigt, dass β-Catenin benötigt wird für die Bildung von sensorischen Zellen und von Melanozyten. In Mausembryonen ohne β-Catenin in den Neuralleisten-Stammzellen wurden keine Spinalganglien gebildet, die aus sensorischen Zellen bestehen. Auch fehlten diesen Mutanten alle Melanozyten.

In der zweiten Publikation wird Wnt/ β-Catenin als instruktives Signal für die Bildung von sensorischen Zellen identifiziert. Neuralleisten-Stammzellen, die ein konstitutiv aktiviertes β-Catenin Protein exprimierten, wurden zu sensorischen Zellen anstelle zu anderen normalerweise durch die Neuralleiste gebildeten Zellen. Zudem wurde in Zellkulturen von einzelnen Neuralleisten-Stammzellen gezeigt, dass sie durch Wnt dazu gebracht wurden sensorischen Zellen zu bilden.

Der dritte Teil der Doktorarbeit beschäftigt sich mit der Frage was für eine Rolle der Wnt/ β-Catenin Signalweg in der Bildung von Melanozyten spielt. Die Tatsache, dass β-Catenin zwar gebraucht wird für die Entstehung von Melanozyten, aber Neuralleisten-Stammzellen ein sensorisches Schicksal wählen wenn sie mit Wnt konfrontiert werden anstatt Melanozyten zu bilden, hat zur Hypothese geführt, dass die Reaktion der Stammzellen auf Wnt Signale abhängig sein könnte vom Zeitpunkt der Konfrontation dieser Neuralleisten-Stammzellen mit Wnt. Indem wir das aktivierte β-Catenin zu verschiedenen Zeitpunkten in Neuralleisten-Stammzellen und in frühen Melanozyten Vorläuferzellen exprimierten, konnten wir zeigen dass β-Catenin nur

innerhalb eines kurzen Zeitfensters fähig ist, die Neuralleisten-Stammzellen in eine Melanozyten- Richtung zu treiben. Diese Resultate stimmen überein mit der Hypothese, dass die Bildung verschiedener von der Neuralleiste abstammender Zelltypen als Reaktion auf Wnt/ β-Catenin Signale abhängig ist vom Zeitpunkt wann diese Signale eintreffen.

3 Introduction

3.1 The neural crest

The neural crest (NC) is transient population of migratory cells unique to vertebrate development. Neural crest cells give rise to a wide array of cell types, including neurons and glia of the peripheral nervous system (PNS), pigment cells in the skin, or bone and cartilage of the head. During embryonic development neural crest cells emigrate from the dorsal neural tube, undergo epithelial to mesenchymal transition (EMT), migrate through the body along distinct pathways and differentiate into the diverse cell types of their respective target sites (Le Douarin et al., 2008; Crane and Trainor, 2006; Ruhrberg and Schwarz, 2010). All these processes are tightly regulated by a complex regulatory network composed of extracellular growth factors and intracellular signaling molecules and transcription factor (Sauka-Spengler and Bronner-Fraser, 2008).

Neural crest populations

The neural crest can be subdivided into at least for different populations according to the rostro- caudal axial level where they delaminate from the neural tube: cranial, vagal, cardiac and trunk neural crest. Cranial neural crest cells leave the neural tube from fore-, mid- and hindbrain regions and generate most of the bone, cartilage and peripheral nerve tissue of the head. Before the cranial neural crest cells build up the complex architecture of craniofacial structures, they temporarily populate the pharyngeal apparatus where they acquire mesenchymal fates (Trainor, 2005; Cordero et al., 2011). Vagal neural crest cells delaminate at the level of somites 1-7

and give rise to the enteric nervous system (ENS) (Heanue and Pachnis, 2007). Colonisation of the gut takes place during embryonic days (E) E9.5 to E15.5. There is a minor contribution of sacral neural crest to the ENS, which originates from very caudal regions of the embryo. The cardiac neural crest gives rise to smooth muscle cells of the cardiac outflow tract and emigrates from neural tube levels of the midotic placode to somite 3 (Sieber-Blum, 2004). Trunk neural crest cells leave neural tube in the trunk region starting from somite 7 and migrate along two separate pathways. Neural crest cells following the ventral pathway between the somites will give rise to sensory neurons and glia of the dorsal root ganglia (DRG), schwann cells along peripheral nerves, autonomic neurons and glia in sympathetic ganglia, and chromaffin cells in adrenal glands. Neural crest cells migrating along the dorsolateral pathway between the somites and the ectoderm will differentiate into pigment cells of the skin (Ruhrberg and Schwarz, 2010; Crane and Trainor, 2006; Huber, 2006).

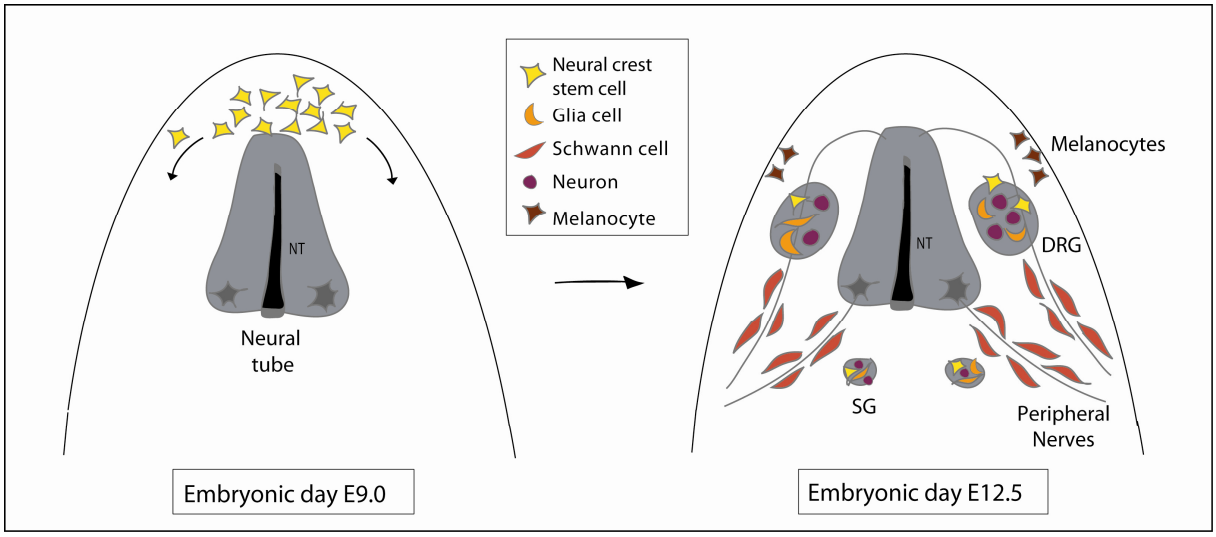

Fig. 1. Derivatives of the trunk neural crest. Schematic representations of transverse sections of a mouse embryo. Neural crest cells in the trunk region leave the neural tube around E9.0 and give rise to sensory cells in dorsal root ganglia (DRG), autonomic cells in sympathetic ganglia (SG), schwann cells along peripheral nerves and melanocytes in the skin.

Neural crest induction and specification

Neural crest cells are born at the neural plate border around the time of neural tube closure. Interactions between the neural and the non-neural ectoderm and signals from the underlying mesoderm lead to induction of the neural crest. An initial gradient of bone morphogenic protein (BMP) in combination with Wnt and fibroblast growth factor (FGF) signaling was shown to be important in neural crest specification (Mayor et al., 1995; Garcia-Castro et al., 2002; Lewis et al., 2004; Monsoro-Burq et al., 2003; LaBonne and Bronner-Fraser, 1998; Steventon et al., 2005; Sauka-Spengler and Bronner-Fraser, 2008). Recent data suggests that earlier events taking place already during gastrulation are important for specification of the neural crest, involving the transcription factor Pax7 (Basch et al., 2006) as well as Wnt and BMP signaling (Steventon et al., 2009). In response to these complex signaling interactions, a unique set of genes is upregulated in the neural plate border region including Zic factors, Pax3/7, Msx1/2 and Dlx3/5 (Nagai et al., 1997; Mansouri et al., 2001; Basch et al., 2006; Catron et al., 1996; Luo et al., 2001). These so-called neural plate border specifier genes will subsequently activate a set of transcription factors in nascent neural crest cells including Snail1, Snail2 (former Slug), AP-2, FoxD3, Sox9, c-Myc and Id (Locascio et al., 2002; Morrison et al., 1991; Dottori et al., 2001; Mori-Akiyama et al., 2003; Bellmeyer et al., 2003; Gammill and Bronner-Fraser, 2003; Meulemans and Bronner-Fraser, 2004; Sauka-Spengler and Bronner-Fraser, 2008). From the onset of migration, neural crest cells express the transcription factor Sox10 and the low-affinity neurotrophin receptor p75 (Paratore et al., 2001; Stemple and Anderson, 1992; Southard-Smith et al., 1998).

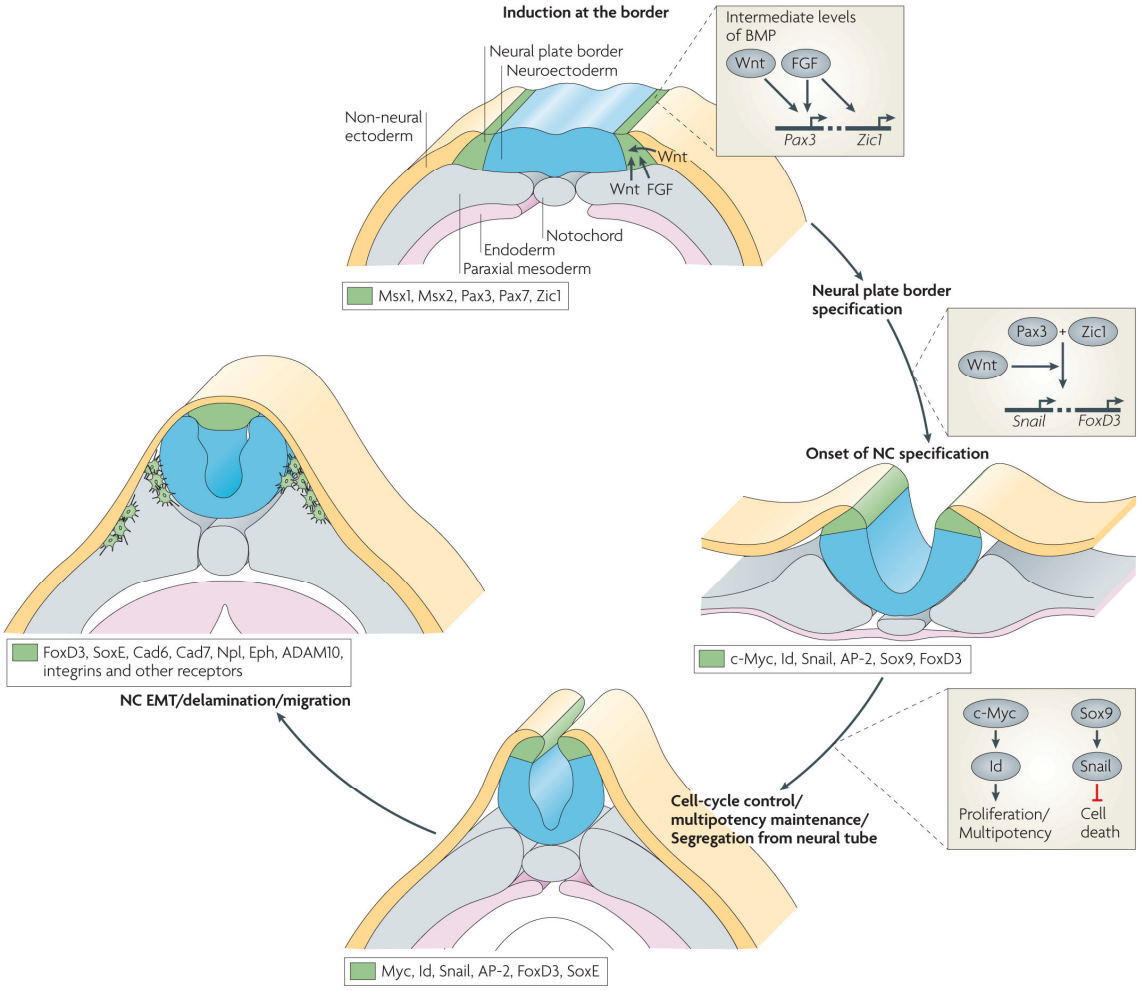

Fig. 2: Regulatory steps in neural crest formation. Combinatorial Wnt-, FGF-, and BMP- signaling specifies the neural crest at the neural plate border. Expression of specific genes in nascent neural crest cells prompts them to undergo EMT and delaminate from the neural tube. Migrating neural crest cells express a distinct set of genes, including Sox10 and p75. Adapted from Sauka-Spengler and Bronner-Fraser, 2008.

Neural crest emigration: epithelial to mesenchymal transition

In order to delaminate from the dorsal neural tube, neural crest cells have to undergo epithelial to mesenchymal transition (EMT), thereby loosing cell-cell adhesion and acquiring migratory capability (Sauka-Spengler and Bronner-Fraser, 2008; Acloque et al., 2009; Kuriyama and Mayor, 2008). EMT is initiated and controlled by extracellular growth factors such as FGF and transforming growth factor (TGF-β)

family members, their downstream signaling pathways and transcriptional regulators including Snail1, Snail2 and FoxD3 (Thiery and Sleeman, 2006). The decrease in cell-cell adhesion properties of neural crest cells is mediated by a switch in Cadherin expression. Type I Cadherins (E-, N-, P- and R- Cadherin) displaying strong adhesiveness are downregulated, instead type II Cadherins (Cadherin 5-12) exhibiting weak adhesive features are expressed (Taneyhill, 2008; Chu et al., 2006; Kuriyama and Mayor, 2008). This, in coordination with Rho GTPase- mediated cytoskeletal changes and cell cycle control enables the neural crest cells to delaminate from the epithelial structure of the neural tube and embark on their migratory pathways (Raftopoulou and Hall, 2004; Liu and Jessel, 1998; Burstyn-Cohen and Kalcheim, 2002). Interestingly, both RhoB and E-Cadherin have been demonstrated to be direct targets of Snail2 (Nieto, 2002; del Barrio and Nieto, 2002; Cano et al., 2000). Migration of neural crest cells is facilitated by digestion of the extracellular matrix (ECM) through the proteolytic activity of matrix metalloproteases (MMP) such as MMP2 and ADAM family members (Cai et al., 2000; Duong and Erickson., 2004; Alfandari et al., 2001).

Neural crest migration

During their migration neural crest cells are guided along specific pathways until they reach their target sites. Surprisingly, restriction to distinct pathways is mainly mediated through repulsive signals, excluding neural crest cells from entering certain regions rather than promoting migration along defined routes (Sauka-Spengler and Bronner-Fraser, 2008). In vertebrate embryos, trunk neural crest cells migrate in metameric fashion only through the anterior half of each somite while avoiding the

posterior half. This pattern of neural crest migration is established and maintained by Neuropilin receptor - Semaphorin ligand interactions and in the chick additionally by the expression of Ephrin family members and their ligands (Gammill and Roffers-Agarwal, 2010; Kuriyama and Mayor, 2008). Chick trunk neural crest cells express a variety of Ephrin receptors, while expression of Ephrin ligands is restricted to the posterior half of each somite. Furthermore, Ephrin receptors and their ligands act as bifunctional guidance cues in the decision between ventral and dorsolateral pathways. They restrict early migrating neural crest cells to the ventral pathway by excluding them from the dorsolateral path, and then later stimulate the migration of melanoblasts into exactly this pathway (Santiago and Erickson, 2002). Similarly, the repulsive ligand Semaphoring 3F (Sema3F) is only expressed in the posterior half of each somite, while trunk neural crest cells express the corresponding receptor neuropilin2 (Npn2) (Gammill et al., 2006). Sema3A is additionally expressed in the dermomyotome, thereby preventing neural crest cells from entering the dorsolateral pathway (Schwarz et al., 2009). Slit-Robo is another receptor- ligand pair involved in neural crest path-finding. Slit2 ligands in the chick dermomyotome restrict Robo1 and Robo2- expressing migrating cells from entering the dorsolateral pathway. Additionally, it prevents them from invading the gut, thus confining them to the ventral pathway (Jia et al., 2005). Attraction of melanoblasts into the dorsolateral pathway is mediated in the chick by differential expression of Endothelin receptors (EDNRs). EDNRB2- expressing melanoblasts are attracted by EDN3 located in the surface ectoderm. Ventrally migrating neural crest cells express EDNRB, the corresponding attractive ligand is unknown (Harris et al., 2008). In contrast, tyrosine kinase receptor Kit seems to regulate neural crest entry into the dorsolateral pathway in the mouse. Kit ligand also known as Steel factor (SLF) or as Stem cell factor (SCF) is expressed

in the dermomyotome. Another example for a chemoattractant that promotes neural crest migration is glial-cell-derived neurotrophic factor (GDNF), which is expressed in the gut mesenchyme and stimulates the migration of ENS progenitors into the intestine (Young et al., 2001).

3.2 Neural crest stem cells (NCSC)

Stem cells are defined by two criteria: multipotency and self-renewal. Multipotency is the ability to give rise to multiple different cell types, while self-renewal leads to the generation of an identical stem cell at every cell division step (Crane and Trainor, 2006).

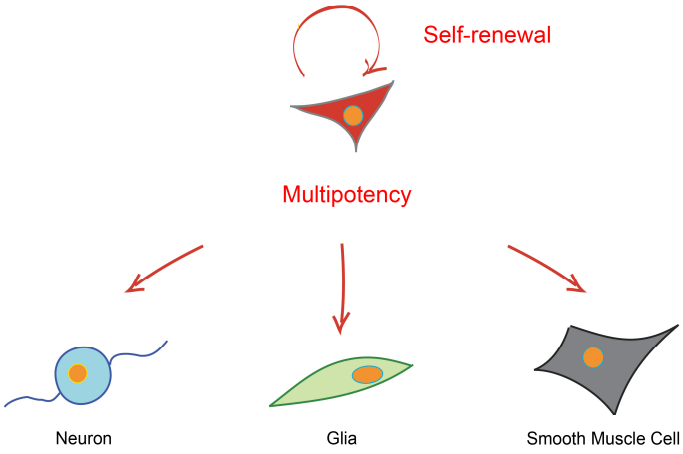

Fig. 3. The two features of stem cells. Self- renewal generates identical daughter cells at every cell division step whereas multipotency is the capacity to differentiate into multiple cell types.

In vivo multipotency or prespecification?

The fact that the neural crest gives rise to many different cell types does not prove the existence of a multipotent neural crest stem cell. The manifold neural crest

derivatives can also be generated through a heterogeneous population of cells already biased towards different lineages at the time of emigration. Subject of an ongoing debate is whether the fate of individual neural crest cells is prespecified in the neural tube or whether they leave the tube as multipotent and multifated cells, and fate restrictions will occur gradually after emigration. There is experimental data in support of both hypotheses. Conflicting results might be explained in part by species- dependent differences in the time- point of neural crest fate specification between chick an mouse (Ruhrberg and Schwarz, 2010; Shakhova and Sommer, 2010).

The existence of a multipotent neural crest stem cell (NCSC) was originally demonstrated in cultures of isolated avian and mouse neural crest cells. When grown at clonal density, individual neural crest cells were able to adopt several different fates (Sieber-Blum and Cohen, 1980; Baroffio et al., 1991; Stemple and Anderson, 1992). Furthermore, in vivo dye injections into single neuroepithelial cells or into emigrating neural crest cells in the avian embryo labelled numerous lineage descendants including sensory neurons, glia and melanocytes (Bronner-Fraser and Fraser, 1988; Bronner-Fraser and Fraser, 1989). Stemple and Anderson proved that isolated neural crest cells are not only multipotent, but in addition also able to self-renew (Stemple and Anderson, 1992). Thus, the emigrating neural crest contains at least some cells which are stem cells and accordingly are referred to as neural crest stem cells (NCSC).

The idea of neural crest prespecification is supported by recent data from the laboratory of Kalcheim and colleagues. Krispin et al. were able to mark single neuroepithelial cells in the chick neural tube and correlate their position in the tube to

the time of delamination and to the fate they adopted (Krispin et al., 2010). Earlier experiments in the mouse identified a subpopulation of neural crest cells that was specified for melanocytic fate already in the dorsal neural tube and expressed the melanoblast marker c-Kit (Wilson et al., 2004). Upon isolation of quail neural crest cells, multipotent cells were found, but they were relatively rare accounting for only 1% of the trunk and 3% of the cranial neural crest cells (Trentin et al., 2004).

Growth factors regulating fate decisions of trunk NCSC

Several growth factors were shown to promote specific fates in cultures of isolated neural crest cells in an instructive way (Le Douarin and Dupin, 2003; Shakhova and Sommer, 2010). Members of the TGF-β family either lead to the generation of smooth muscle cells, autonomic neurons or apoptosis, dependent on the context (Hagedorn et al., 1999). Upon addition of BMP-2 many neural crest cells adopted an autonomic neuron fate (Morrison et al., 1999) while Neuregulins (NRG) prompted the cells to acquire glial fate (Shah et al., 1994). Only recently and in the frame of this thesis Wnt1 has been identified as an instructive signal for the generation of sensory neurons (Lee et al., 2004).

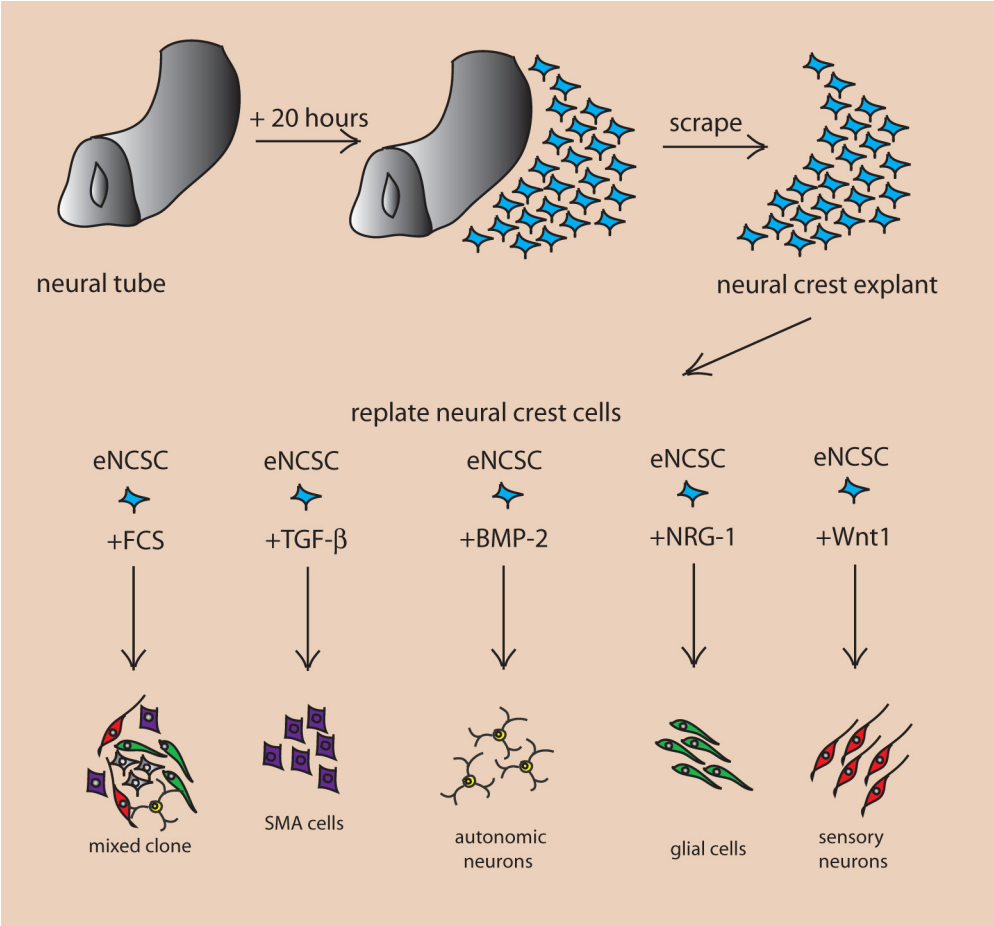

Fig. 4 Fate decisions of NCSC are regulated by instructive growth factors. Schematic representation of an in vitro culture system used to isolate NCSC and to identify extracellular factors involved in neural crest fate decisions. Instructive growth factors promote the adoption of specific fates at the expense of other possible lineages. Adapted from Shakhova and Sommer, 2010.

Postmigratory NCSC

Despite the fact that the neural crest is a transient structure restricted to embryonic development NCSC-like cells have also been isolated from postmigratory neural crest targets such as the sciatic nerve, the DRG and the gut (Morrison et al., 1999; Bixby et al, 2002). Surprisingly, even adult tissues were demonstrated to harbour cells with NCSC- like potentials. Such cells were isolated for example from the adult skin, the cornea and the bone marrow (Wong et al., 2006; Yoshida et al., 2006; Nagoshi et al., 2008).

3.3 Wnt/ β-Catenin signaling in neural crest development

Wnt signaling pathways

Wnt signaling is a fundamental mechanism involved in many processes during embryonic development and in adult tissue homeostasis. Abnormal Wnt signaling is associated with various human diseases including cancer (Moon et al., 2004; Huang and He, 2008, Grigoryan et al., 2008). The Wnt family of secreted lipoproteins and their drosophila homologue wingless (Wg) regulate cell proliferation, cell polarity and cell fate determination. 19 mammalian Wnt homologues have been found to date, they are expressed in overlapping temporal- spatial patterns. Wnt protein processing and secretion is a highly regulated process. Wnts are glycosylated and lipid modified in the endoplasmic reticulum (ER), a step involving Porcupine (Porc). Subsequently, they are escorted to the plasma membrane for secretion by Wntless (Wls). Wnts can act as short- or long-range morphogens. In drosophila, transport of hydrophobic Wnt proteins over long distances is presumably facilitated by large lipoprotein particles (Mikels and Nusse, 2006; Port and Basler, 2010).

Three main pathways can be activated in response to binding of Wnt to its Frizzled (Fz) receptor: the canonical Wnt/ β-Catenin cascade (Fig5., blue) the noncanonical Jun kinase or planar cell polarity (PCP) pathway (Fig5., green) and the Wnt/ Ca2+ pathway (Fig5., yellow). The best- studied of these pathways is the highly conserved canonical Wnt/ β-Catenin signaling pathway. It regulates the stability of the transcription cofactor β-Catenin (the vertebrate homologue of drosophila armadillo) and thereby controls β-Catenin-dependent gene expression (Logan and Nusse, 2004; Clevers 2006; McNeill and Woodgett, 2010). In the Jun kinase pathway, Dishevelled (Dsh) regulates cytoskeletal organisation and cell polarity via the

activation of small GTPases such as Rho and Rac. The Wnt /Ca2+ pathway leads to release of intracellular calcium and activation of Phospholipase C (PLC) and of protein kinase C (PKC) (Sugimura and Li, 2010; Grigoryan et al., 2008). Members of the different pathways interact with each other, rather representing a complex intracellular signaling network than three independent cascades (Kestler and Kuhl, 2008).

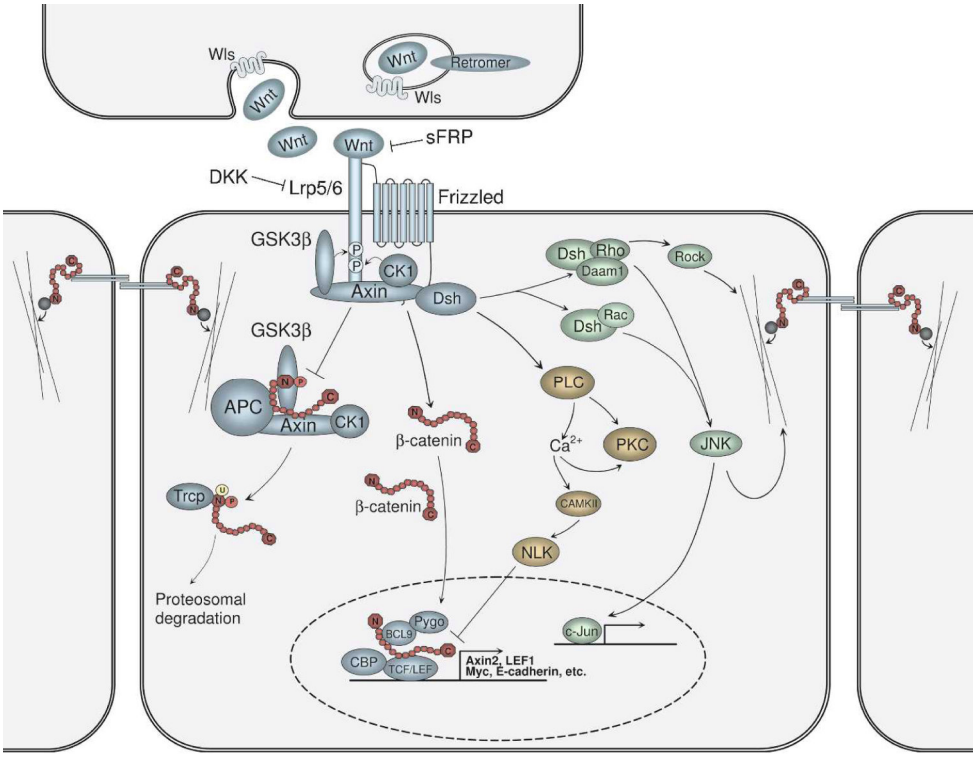

Fig. 5. Wnt signaling pathways. The canonical pathway (blue) signals by regulating the cytosolic pool of β-Catenin. The non canonical Jun kinase pathway (green) acts via small Rho- GTPases. The noncanonical Wnt/ Ca2+ pathway (yellow) leads to release of intracellular calcium and activation of Phospholipase C (PLC) and of protein kinase C (PKC). The three pathways branch at the level of Dishevelled (Dsh). Adapted from Grigoryan et al., 2008.

Canonical Wnt signaling involves β- Catenin

In the absence Wnt, cytosolic levels of β-Catenin are maintained low. Degradation of β-Catenin is regulated by the destruction complex composed of adenomatous polyposis coli gene product APC, Axin1, glycogen synthase kinase 3 (GSK3), and casein kinase 1 (CK1), which binds and phosphorylates β-Catenin. Phosphorylation targets β-Catenin for ubiquitinylation and subsequent degradation by the proteasome. Following Wnt binding, the Fz receptor is thought to form a co-receptor complex with low-density-lipoprotein receptors LRP5 or LRP6. This interaction leads to phosphorylation of the LRP5/ 6 intracellular domains, thus creating a binding site for Axin2. By recruitment of Axin2 to the plasma membrane, the destruction complex is disrupted and phosophorylation of β-Catenin omitted. Consequently, β-Catenin accumulates in the cytosol, translocates to the nucleus and regulates transcription of specific genes together with lymphoid enhancer-binding factor 1/ T cell- specific transcription factors (LEF/ TCF). In the absence of Wnt signaling, TCF acts as a repressor of Wnt target genes by forming a complex with members of the Groucho/ TLE familiy of transcriptional co-repressors. Upon canonical Wnt signaling, β-Catenin interferes with the TCF- Groucho interaction and activates gene expression together with TCF and CREB- binding protein (CBP). β-Catenin also binds Legless (Bcl9) and Pygopus (Pygo), proteins which are presumably involved in chromatin remodelling that favours gene transcription (Mac Donald et al., 2009; Clevers 2006; Logan and Nusse, 2004).

In addition to being a central component of the Wnt signaling pathway β-Catenin can also participate in cell- cell adhesion in a complex with Cadherin cell adhesion molecules (Nelson and Nusse, 2004).

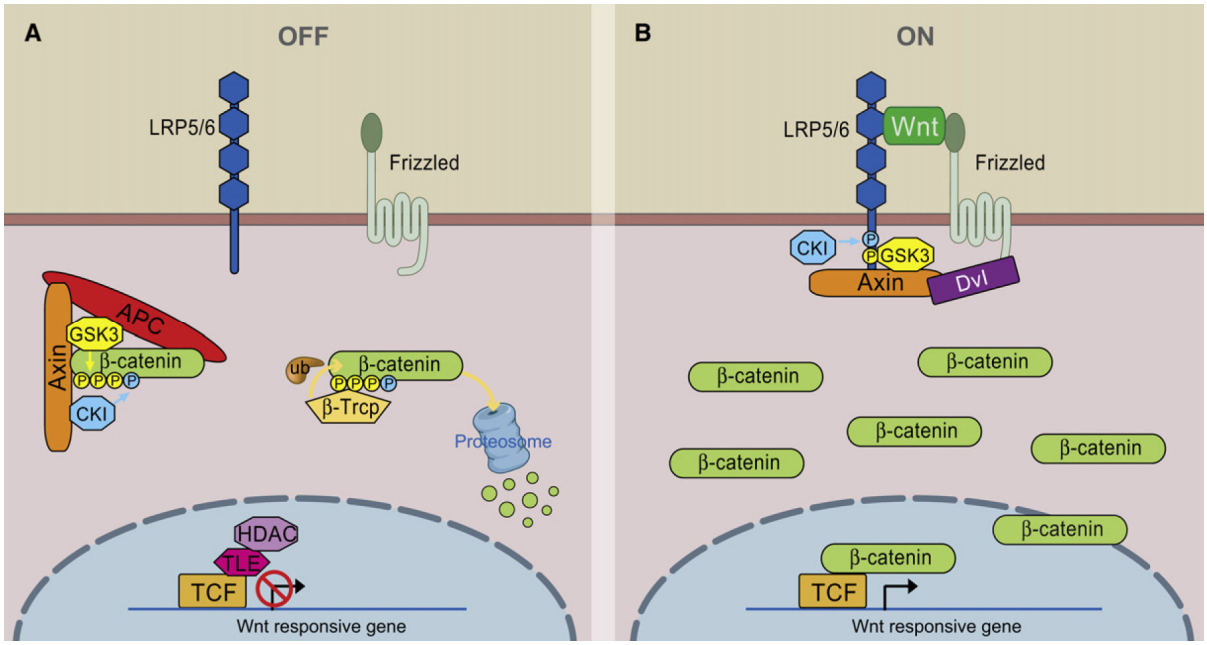

Fig. 6. The canonical Wnt signaling pathway. (a) In the absence of Wnt, cytosolic β-Catenin is targeted for degration by the destruction complex containing APC, Axin1, GSK3, and CK1. (b) Upon binding of a Wnt ligand to its Frizzled and LRP receptors, the destruction complex is disrupted, β-Catenin accumulates in the cytosol and translocates to the nucleus where it regulates transcription of specific genes. Adapted from MacDonald et al., 2009.

Wnt/ β- Catenin function in the sensory lineage

Wnt signaling plays multiple roles in neural crest development. First, it is necessary for induction of the neural crest at the neural plate border. Later, together with BMP growth factors, it is able to maintain NCSC in an undifferentiated state (Kleber et al., 2005). Furthermore, Wnt signaling is involved in generation of sensory and melanocyte lineages.

The neural crest- derived sensory lineage consists of sensory neurons and associated glia located in the DRG. Some neural crest- derived sensory cells also contribute to formation of cranial ganglia V, VII, IX and X. Neurogenesis in the DRG occurs in three waves. A first wave initiates early at the time of neural crest emigration and is defined by the expression of the basic helix-loop-helix transcription

factor Neurogenin (Ngn) 2. In a second wave occurring upon DRG condensation, sensory cells are specified by expression of Ngn1 (Ma et al., 1999). Boundary cap cells give rise to a small number of sensory neurons in a third wave of neurogenesis (Maro et al., 2004).

Subtypes of sensory neurons in the DRG are characterized by the expression of neurotrophin receptor tyrosine kinases (Trk) A, B or C. Large diameter mechanoreceptive and proprioceptive TrkB- and TrkC- positive neurons are mainly derived from Ngn2- expressing sensory progenitors. Ngn1- positive cells will generate the majority of small diameter nociceptive TrkA- positive neurons, but also some TrkB and TrkC positive neurons, as in Ngn2 knock out mice all types of sensory neurons exist. In contrast, Ngn1- deficient embryos display a complete loss of TrkA- positive neurons (Ma et al., 1999; Marmigere and Ernfors, 2007).

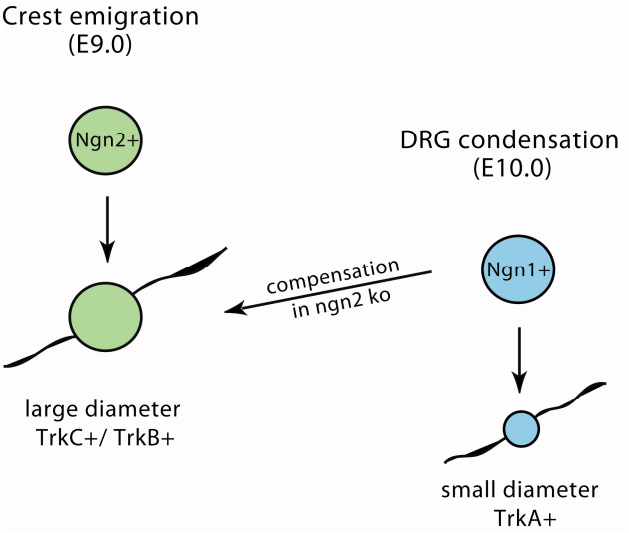

Fig. 7. Two types of sensory neurogenesis in the DRG. Ngn2-positive sensory progenitors are specified at the time of neural crest emigration and later give rise to TrkC- and TrkB- positive neurons. Ngn1 expression starts upon DRG condensation, these cells will later give rise to TrkA- positive neurons but are also able to compensate for the loss of Ngn2-depdendent neurogenesis in Ngn2- deficient embryos.

First indications of an important role of Wnt signaling in generation of the sensory lineage came from analysis of Wnt1/ Wnt3a double knock out animals. In Wnt1/ Wnt3a compound mutants, the sensory lineage was completely lost (Ikeya et al., 1997). Results obtained in our lab are consistent with these findings and will be

presented in the first two publications of this thesis. The first publication demonstrates requirement of Wnt/ β-Catenin signaling for specification of the sensory lineage. Analysis of mouse embryos lacking β-Catenin specifically in the neural crest revealed complete absence of the DRG. Moreover, neither Ngn2- positive cells shortly after neural crest emigration, nor Ngn1- positive cells at the time of DRG condensation were present.

In the second part of my thesis we show that β-Catenin is not only required, but also sufficient to drive neural crest cells into the sensory lineage. We identified Wnt1 as an instructive growth factor on early NCSC for sensory lineage specification. Analysis of mouse embryos expressing a constitutively activated form of β-Catenin in the neural crest population, and of cultures of isolated NCSC exposed to Wnt demonstrated that NCSC chose a sensory fate in response to canonical Wnt signaling at the expense of other fates.

Wnt/ β- Catenin function in the melanocyte lineage

Analysis of β-Catenin- deficient embryos not only demonstrated requirement of β-Catenin for specification of the sensory lineage, but also for generation of melanocytes (first publication of my thesis, Hari et al., 2002). Further support for a prerequisite of Wnt/ β-Catenin signaling in the formation of melanocytes came from experiments done in zebrafish and in cultures of avian and mouse neural crest cells. Upregulation of Wnt- controlled genes by injection of *β-catenin* mRNA into single neural crest cells in zebrafish promoted pigment cell formation at the expense of neurons and glia (Dorsky et al., 1998). Quail neural crest cells cultured in the

presence of Wnt3a preferentially gave rise to pigment cells while numbers of neuronal or glial cells were decreased (Jin et al., 2001). Similarly, in mouse neural crest cultures, overexpression of wnt1, wnt3a, and β-catenin in melanoblasts and in neural crest cells resulted in expansion of the melanoblast population and in accelerated differentiation of pigment cells (Dunn et al., 2000; Larue et al., 2003; Sommer, 2011,).

The effect of Wnt signaling on melanocyte fate specification is possibly mediated by microphtalmia- associated transcription factor Mitf. Expression of Mitf is thought to be a key event in melanocyte specification. It marks melanoblasts shortly after emigration, regulates expression of several genes involved in melanogenesis, and controls melanoblast development and survival (Opdecamp et al., 1997; Thomas and Erickson, 2008). Mitf has been shown to be a direct target of Wnt3a via binding of LEF1 to the mitf- promoter (Takeda et al., 2000; Levy et al., 2006).

Formation of melanoblasts is also regulated by the transcription factors Pax3, Sox10 and FoxD3, which are presumably controlled by Wnt signaling (Meulemans and Bronner-Fraser, 2004). Pax3 and Sox10 were shown be able to bind synergistically to the *mitf* promoter region and to activate expression of *mitf* (Bondurand et al., 2000). Pax3 alone is not required for melanoblast specification, but for expansion of the melanoblast pool at early developmental stages. In Pax3 mutant embryos, melanoblasts are still present but reduced in numbers (Hornyak et al., 2001). In contrast, Sox10 seems to be required for melanocyte fate specification, since Sox10-deficient embryos lack all melanocytes (Bondurand et al., 2000; Britsch et al., 2001). FoxD3, on the other hand, represses melanogenesis and transcription of *mitf*. FoxD3 expression starts in the dorsal neural tube, is maintained in migrating glial and neural

precursors, but downregulated in melanoblasts. Downregulation of FoxD3, however, is not sufficient for melanocyte specification (Kos et al., 2001; Thomas and Erickson, 2009).As soon as melanocytes precursors called melanoblasts embark on the dorsolateral pathway they are characterized by the expression of Mitf, c-Kit, dopachrome tautomerase (Dct) and later tyrosinase-related-protein 1 (Trp1) and Tyrosinase, the rate- limiting enzyme in melanin synthesis. SCF and EDN growth factors are required for the correct dorsolateral migration of melanoblasts. Both factors are additionally involved in survival, proliferation and differentiation of melanoblasts, but not in initial melanocyte fate specification (Wehrle-Haller and Weston, 1995; Hou et al., 2000; Saldana-Caboverde and Kos, 2010; Sommer, 2011).

In the third part of my thesis I investigated the role of canonical Wnt/ β-Catenin signaling in melanocyte fate acquisition. The fact that β-Catenin is required for formation of the melanocyte lineage but NCSC in the presence of Wnt chose a sensory rather than a melanocytic fate brought up the idea of a stage- dependent response of NCSC to Wnt. By expressing a stabilized form of β-Catenin at different stages of neural crest and early melanocyte development we demonstrated that β-Catenin activation can promote the generation of melanoblasts only during a narrow time window of neural crest development.

Tools to manipulate Wnt/ β-Catenin signaling in neural crest development

In order to circumvent early embryonic lethality of *β- catenin* knock out embryos, we conditionally inactivated *β- catenin* using the *Cre/loxP* system (Gu et al., 1993; Haegel et al., 1995; Brault et al., 2001). The same strategy was used to generate a

stabilized form of β-Catenin specifically in neural crest cells (Harada et al., 1999; Grigroyan et al., 2008).

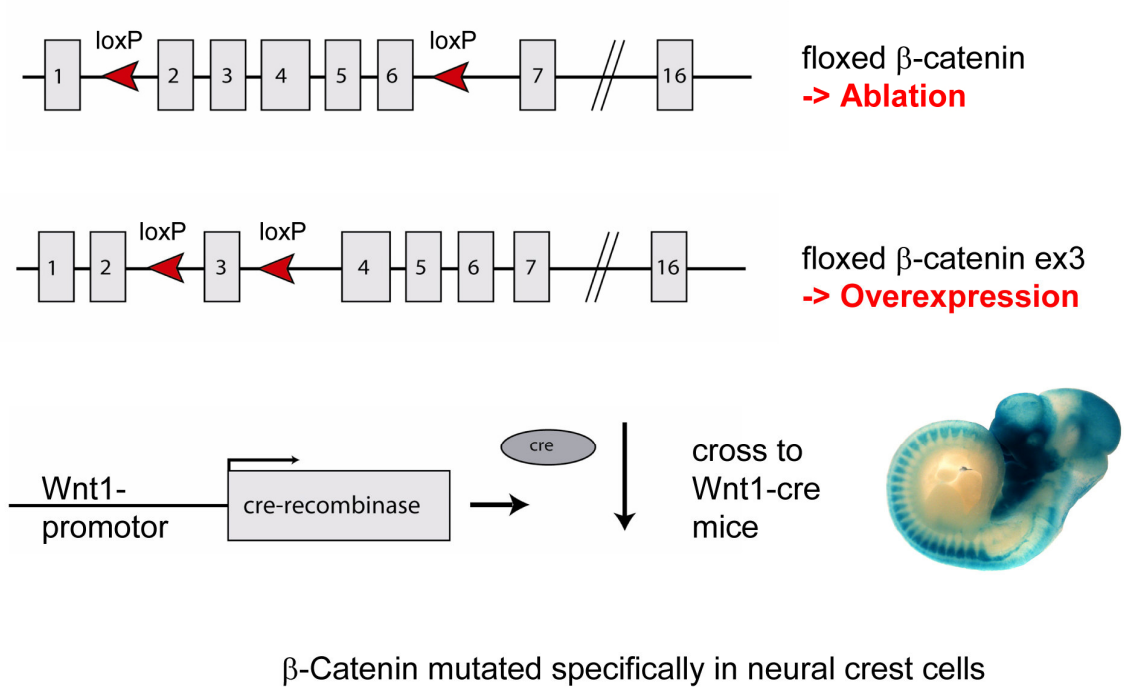

Fig. 8 Manipulation of β-Catenin in NCSC using the Cre/loxP system. Schematic representation of the gene constructs used to either ablate or stabilize β-Catenin. *Wnt1-Cre* mice were used to specifically mutate *β-catenin* in premigratory neural crest cells and their progeny. An X-Gal wholemount of an E10.5 *Wnt1-Cre* embryo crossed to the R26R reporter strain illustrates recombination of neural crest- derived cells.

To manipulate *β-catenin* in neural crest cells at different time-points, we used several Cre-lines with distinct Cre- expression patterns. The *Wnt1-Cre* line was used to manipulate *β-catenin* at very early or even premigratory stages (Danielian et al., 1998), *Sox10-Cre* lead to mutation of *β-catenin* in migratory neural crest cells (Matsuoka et al., 2005), stabilization of β-Catenin in glial progenitors was achieved by using the *Dhh-Cre* line (Jaegle et al., 2003) and the *Plp-CreERT2* line (Leone et al., 2003), while expression of a constitutively active form of β-Catenin in melanoblast was obtained using the *Tyr-CreERT2* line (Bosenberg et al., 2006). CreERT2 is a

fusion protein of a mutated ligand-binding domain of the estrogen receptor and the Cre recombinase. Activation of the CreERT2 recombinase can be temporally controlled by administration of tamoxifen. Lineage tracing of recombined cells was monitored by crossings to ROSA26 reporter (R26R) (Soriano, 1999) or Z/EG (Novak et al., 2000) mouse lines where recombination leads to expression of either β-Galactosidase or EGFP protein, respectively.

4 Results

4.1 Lineage-specific requirements of β-*catenin* in neural crest development

J. Cell Biol. **159**: 867-880; 2002

My contribution to this work:

- Designing and performing the experiments presented in Fig. 1, 2, 3E-L, 6, 7, 8A-C
- Collection of the data for Fig. 1, 2, 3E-L, 6, 7, 8A-C
- Generation of Fig. 1, 2, 3E-L, 6, 7, 8A-C
- Generation of most of the reagents; embryos were kindly provided by V. Brault
- Design of the paper outline, together with L. Sommer. Writing of “Figure Legends” and “Materials and Methods”. Proofreading of the manuscript.

JCB Article

Lineage-specific requirements of β-*catenin* in neural crest development

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β-Catenin plays a pivotal role in cadherin-mediated cell adhesion. Moreover, it is a downstream signaling component of Wnt that controls multiple developmental processes such as cell proliferation, apoptosis, and fate decisions. To study the role of *β-catenin* in neural crest development, we used the *Cre/loxP* system to ablate *β-catenin* specifically in neural crest stem cells. Although several neural crest–derived structures develop normally, mutant animals lack melanocytes and dorsal root ganglia (DRG). In vivo and in vitro analyses revealed that mutant neural crest cells emigrate but fail to generate an early wave of sensory neurogenesis that is normally marked by the transcription factor neurogenin (ngn) 2. This indicates a role of *β-catenin* in premigratory or early migratory neural crest and points to heterogeneity of neural crest cells at the earliest stages of crest development. In addition, migratory neural crest cells lateral to the neural tube do not aggregate to form DRG and are unable to produce a later wave of sensory neurogenesis usually marked by the transcription factor ngn1. We propose that the requirement of *β-catenin* for the specification of melanocytes and sensory neuronal lineages reflects roles of β-catenin both in Wnt signaling and in mediating cell–cell interactions.

Introduction

Multipotent stem cells have to generate various differentiated cell types in the correct number and sequence during neural development (Sommer and Rao, 2002). The neural crest has turned out to be a valuable model system to study the mechanisms controlling this process. Neural crest cells in vertebrates give rise to neuronal and glial cells of the peripheral nervous system (PNS)* and generate nonneural cells such as pigment and smooth muscle–like cells. Many signals have been described that promote the formation of particular cell fates in migratory and postmigratory neural crest stem cells (for reviews see Anderson et al., 1997; Dorsky et al., 2000a; Sommer, 2001). Melanocyte formation is induced by Wnt signaling (Dorsky et al., 1998; Jin et al., 2001), whereas TGFβ promotes the development of smooth muscle–like cells (Shah et al., 1996). Notch signaling and NRG1 isoforms promote the generation of satellite glia and Schwann cells (Shah et al., 1994; Hagedorn et al., 2000b; Morrison et al., 2000; Leimeroth et al., 2002), whereas members of the TGFβ family promote the differentiation of autonomic neurons (Reissmann et al., 1996; Shah et al., 1996; Hagedorn et al., 2000a). Signals specifying sensory neurons from neural crest cells, however, have not yet been reported. There is increasing evidence that the fate of multipotent neural crest stem cells is not only dependent on the action of individual signals but is also influenced by the synergistic activity of multiple signals (Sommer and Rao, 2002). Hitherto unknown signals provided by cell–cell interactions alter the biological activity of instructive growth factors such as TGFβ, indicating that neural crest cells are able to integrate multiple cues (Hagedorn et al., 1999, 2000a). Moreover, cell-intrinsic changes affect fate decisions during neural crest development by changing the sensitivity of neural crest cells to specific extracellular signals (Paratore et al., 2001, 2002; White et al., 2001; Kubu et al., 2002).

β-Catenin is a good candidate to be involved in lineage decisions in neural crest development, given its dual function in cadherin-dependent cell–cell interactions and in mediating Wnt signaling, and that both cadherins and Wnt molecules have been implicated in neural development (Cadigan and Nusse, 1997; Yagi and Takeichi, 2000). By binding to the cytoplasmic domain of cadherins and to α-catenin, β-catenin

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*Abbreviations in this paper: bHLH, basic helix-loop-helix; DRG, dorsal root ganglia; E, embryonic day; mitf, microphtalmia-associated transcription factor; ngn, neurogenin; nf, neurofilament; PNS, peripheral nervous system; trk, tyrosine receptor kinase; trp2, tyrosinase-related protein 2.

Key words: β-catenin; Wnt; cadherin; melanocytes; sensory neurons

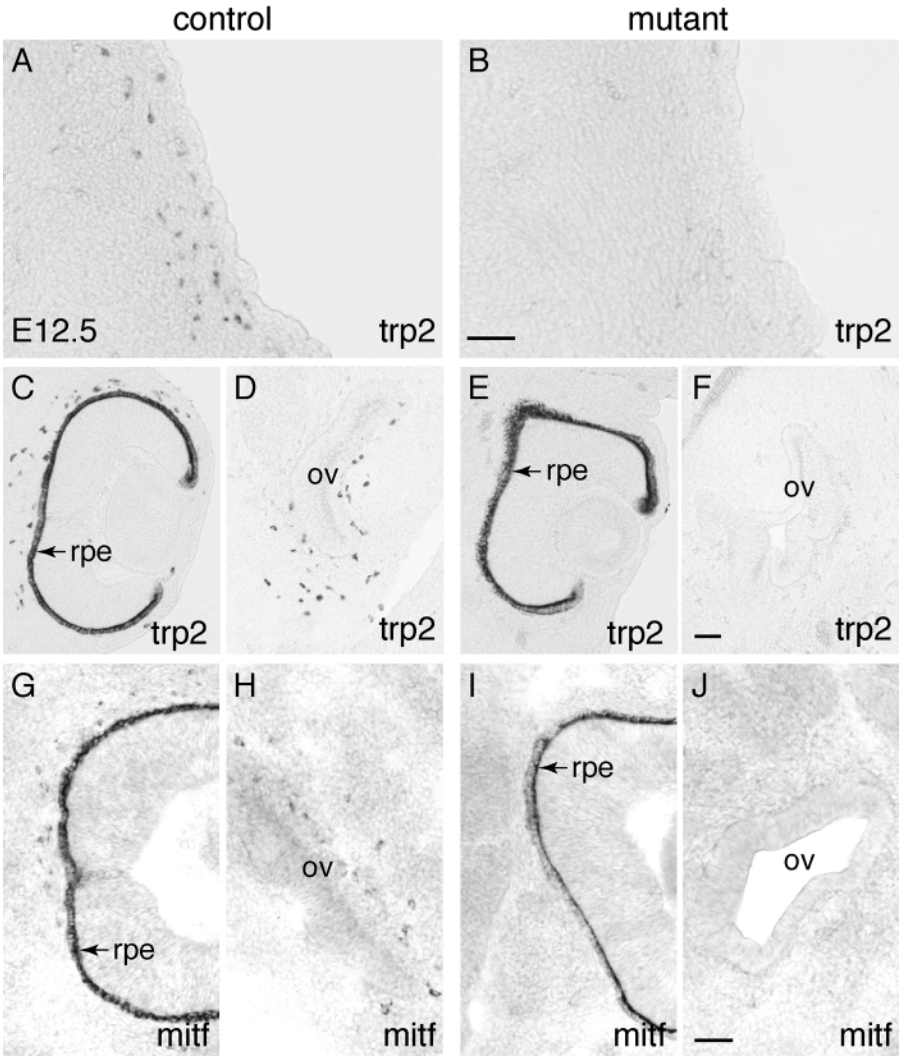


Figure 1. **Absence of melanocytes in *β-catenin* mutant embryos.** Melanocytes and their precursors were marked by in situ hybridization analysis on transverse sections of E12.5 control (A, C, D, G, and H) and mutant (B, E, F, I, and J) embryos using trp2 (A–F) and mitf (G–J) riboprobes. In mutant embryos, trp2-positive melanocytes were absent underneath the surface ectoderm (B), in areas surrounding the retinal pigment epithelium (rpe, E), and around the otic vesicle (ov, F), whereas many melanocytes were present in control embryos (A, C, and D). Many mitf-expressing melanoblasts were found around the eye (G) and the otic vesicle (H) of control but not mutant embryos (I and J). Bars, 50 μm.

links the cadherin-dependent adhesion complex to the cytoskeleton and thereby strengthens cellular interactions (Vleminckx and Kemler, 1999; Gumbiner, 2000). Wnt signaling has been implicated in the regulation of cell proliferation, apoptosis, and cell fate decisions (Cadigan and Nusse, 1997; Uusitalo et al., 1999). Activation of this pathway leads to the stabilization of β-catenin and to its translocation into the nucleus, where it associates with other nuclear effectors to form a transcriptional activator complex. In various biological systems, Wnt/β-catenin signaling has been identified as part of a signaling network that involves interactions with other signal transduction pathways such as TGFβ and Notch signaling (Hecht and Kemler, 2000; De Strooper and Annaert, 2001). A putative role of β-catenin in neural crest development is thus likely to reflect functions of cadherin-mediated adhesion and Wnt signaling. Cadherins influence neural crest specification and emigration from the neural tube (Nakagawa and Takeichi, 1998; Borchers et al., 2001). Moreover, the expression of cadherins in aggregating dorsal root ganglia (DRG) and in Schwann cells is consistent with a role at later stages of neural crest development (Pla et al., 2001). Wnt signaling is involved early in neural crest development and regulates neural crest induction and expansion (Ikeya et al., 1997; Saint-Jeannet et al., 1997; LaBonne and Bronner-Fraser, 1998), apart from the generation of melanocytes mentioned above.

To investigate the role of β-catenin in neural crest stem cells and their derivatives, we performed conditional gene ablation of *β-catenin* in premigratory neural crest. Such an approach circumvents the early embryonic lethality of mutants generated by gene deletion in the germ line (Haegel et al., 1995; Huelsken et al., 2000) and has been successfully applied before to reveal a requirement of *β-catenin* in brain and craniofacial development, skin stem cell differentiation, and fate decisions between endoderm and precardiac mesoderm (Brault et al., 2001; Huelsken et al., 2001; Lickert et al., 2002). The present study identifies β-catenin as a crucial regulator of sensory neuron specification and melanocyte formation.

Results

Inactivation of *β-catenin* in neural crest

The role of *β-catenin* in neural crest development was addressed by conditional gene inactivation in neural crest stem cells using the *Cre/loxP* recombination system (Gu et al.,

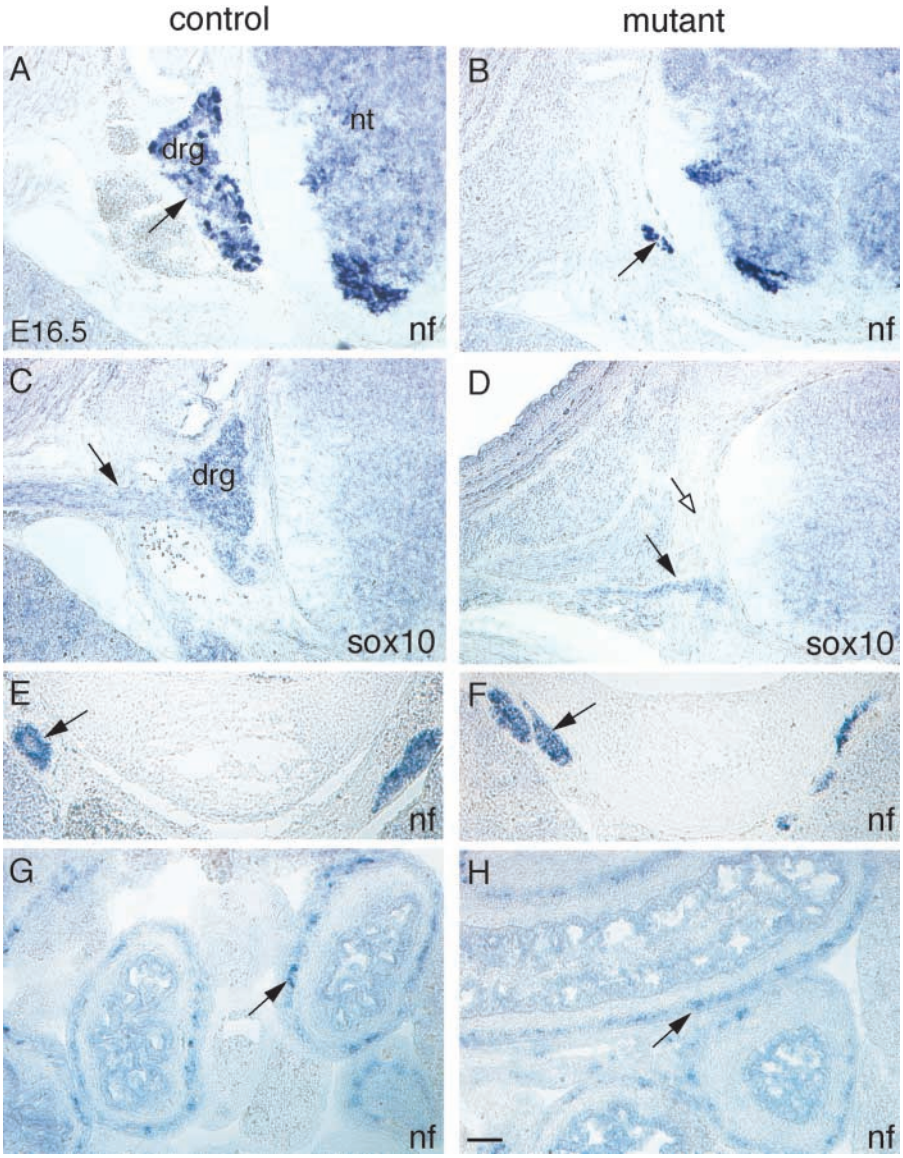


Figure 2. **Analysis of control and mutant PNS.** In situ hybridization experiments on transverse sections of E16.5 embryos with nf (A and B and E–H) and sox10 (C and D) riboprobes revealed a reduction of neuronal (A and B, arrows) and complete absence of glial (C and D, open arrow) lineages in dorsal root ganglia (drg). Peripheral nerves marked by sox10 (C and D, arrows) were reduced in diameter, whereas other crest derivatives, such as sympathetic ganglia (E and F, arrows) and the enteric nervous system (G and H, arrows), appeared to develop normally. nt, neural tube. Bar, 50 μm.

1994). Cre-mediated recombination of a floxed allele in which essential sequences of the *β-catenin* gene are flanked by *loxP* sites generates the *β-catenin* floxdel allele, from which no functional β-catenin protein is expressed (Brault et al., 2001). In *wnt1-Cre* mice, Cre recombinase is active in the entire neural crest population (Danielian et al., 1998). To generate neural crest–specific *β-catenin* mutant embryos, we crossed *wnt1-Cre* animals heterozygous for the *β-catenin* floxdel allele with animals homozygous for the *β-catenin* floxed allele. In *wnt1-Cre*/*β-catenin*$^{floxed/floxdel}$ embryos derived from such breeding, β-catenin expression is efficiently eliminated in virtually all neural crest stem cells (Brault et al., 2001). In contrast, littermates lacking the *wnt1-Cre* transgene or carrying a wild-type *β-catenin* allele express β-catenin normally and serve as control animals.

Loss of the melanocyte lineage in *β-catenin* mutant embryos

We first investigated the role of *β-catenin* in melanocyte development. Neural crest cells give rise to melanocytes of the skin, inner ear, and part of the iris (Wehrle-Haller and Weston, 1997). Wnt signaling promotes melanocyte formation from neural crest cells and absence of *wnt1* and *wnt3a* in compound mutant mice results in the loss of the melanocyte differentiation marker tyrosinase-related protein 2 (trp2) (Ikeya et al., 1997; Dorsky et al., 1998; Dunn et al., 2000; Jin et al., 2001). Similarly, we observed a complete absence of trp2 expression in *β-catenin* mutant embryos at embryonic day (E) 10.5 and E12.5 (Fig. 1; unpublished data). This phenotype was apparent at all sites of neural crest–derived melanocyte formation, such as underneath the surface ectoderm (Fig. 1, A and B), around the retinal pigment epithelium (Fig. 1, C and E), and around the otic vesicle (Fig. 1, D and F). In contrast, trp2-positive cells in the retinal pigment epithelium, which are not generated from neural crest, were not affected in the mutant.

To address whether the lack of trp2 expression reflects a requirement for *β-catenin* in early or late melanocyte differentiation, the expression of microphtalmia-associated transcription factor (mitf) was analyzed. Mitf activates pig-

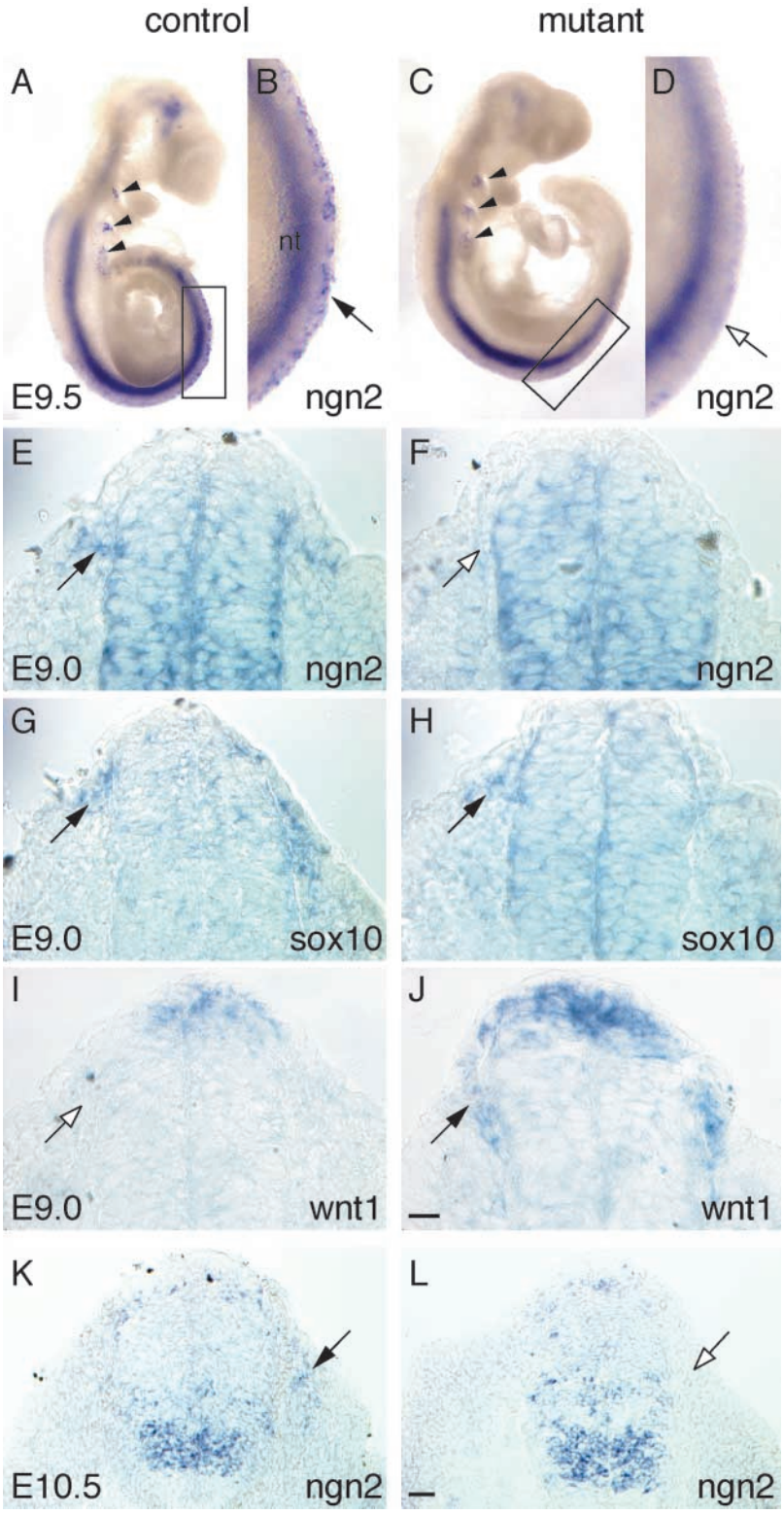
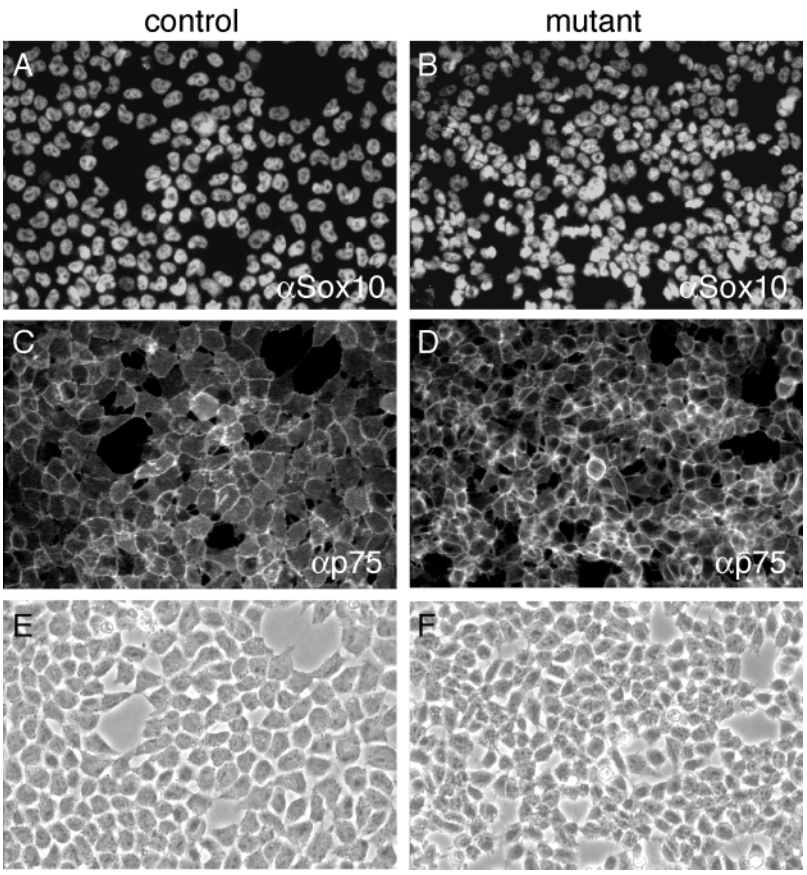


Figure 3. **Absence of the ngn2-expressing sensory sublineage in the emigrating crest of mutant embryos.** Whole mount in situ hybridization experiments showed ngn2 expression in the neural tube and in placodes (arrowheads) of control and mutant embryos at E9.5 (A–D). In migratory crest, ngn2 mRNA was only detectable in control (A and B, arrow) but not in mutant embryos (C and D, open arrow). The boxes in A and C indicate the areas enlarged in B and D, respectively. On transverse sections at E9.0, ngn2-positive neural crest cells were found in control (E, arrow) but not in mutant (F, open arrow) embryos. In contrast, on adjacent sections, neural crest emigrating from the dorsal neural tube was marked by sox10 mRNA both in control and mutant embryos (G and H, arrows). As at earlier stages, ngn2 mRNA was present in the DRG anlage of control (K, arrow) but not of mutant embryos (L, open arrow) at E10.5. Hybridization with a wnt1 riboprobe showed maintained wnt1 expression in the emerging crest of mutant embryos (J, arrow), whereas it was down-regulated in neural crest of control embryos (I, open arrow) at E9.0. Bars: (E–J) 10 μm; (K and L) 20 μm.

ment cell–specific genes such as *trp2* and is required for promoting melanoblast formation from precursor cells (Opdecamp et al., 1997; Yasumoto et al., 1997). In contrast to control embryos (Fig. 1, G and H), mitf-positive melanoblasts were not detectable in *β-catenin* mutant embryos (Fig. 1, I and J), indicating that melanoblasts never formed in the mutant.

Analysis of peripheral neural structures

Our initial analysis of embryos in which *β-catenin* had been eliminated by *wnt1-Cre*–mediated gene ablation previously revealed a reduction of neuronal cells in cranial ganglia and DRG at E10.5 (Brault et al., 2001), suggesting a role for *β-catenin* in the formation of peripheral neurons. To elucidate this further, we first analyzed the PNS of mutant ani-



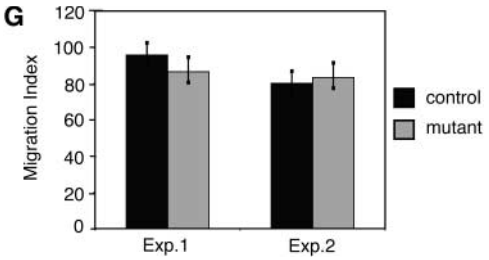


Figure 4. **Normal emigration of mutant neural crest cells.** Neural crest explants were obtained from neural tubes that had been isolated from control and *β-catenin* mutant mice at E9 and cultured for 20 h to allow emigration of neural crest cells. After emigration, neural crest cells were fixed and immunolabeled with anti-sox10 antibody (visualized by Cy3 fluorescence) (A and B) and anti-p75 antibody (visualized by FITC fluorescence) (C and D). Note that virtually all neural crest cells were double positive for the neural crest stem cell markers p75 and sox10. (E and F) Corresponding phase contrast pictures. (G) To compare and quantify the extent of control and mutant neural crest outgrowth after 20 h, the migration index was calculated using the NIH image 1.62 software (Materials and methods). Two independent experiments using nonsibling embryos were performed, scoring three explants of control and mutant embryos per experiment. Each bar represents the migration index (mean ± SD) of three different explants. Note that mutant explants were not significantly reduced in size and density.

mals at a late embryonic stage. Only a few occasional neurons marked by neurofilament (nf) 160 were detectable in the mutant as compared with the control in DRG of E16.5 embryos (Fig. 2, A and B). In contrast, sympathetic ganglia and the enteric nervous system were not affected (Fig. 2, E–H). To address whether the phenotype in mutant DRG was due to a specific requirement for *β-catenin* in neuronal differentiation, we analyzed the expression of the transcription factor sox10. In the PNS, sox10 is a marker for multipotent progenitors and glial cells whose expression is down-regulated as progenitors adopt a neuronal or nonneural fate (Paratore et al., 2001). Although control DRG and peripheral nerves were composed of many sox10-positive cells, we were unable to detect any progenitor cells or presumptive satellite glia lateral to the neural tube where DRG normally form (Fig. 2, C and D). Thus, only residual DRG were present in E16.5 mutant embryos, consisting of a few neuronal cells without associated progenitor cells or glia. As expected, given the near absence of sensory axons, mutant peripheral nerves were marked by sox10 but were reduced in size (Fig. 2, C and D). The absence of *β-catenin* apparently did not impair early Schwann cell differentiation, as Schwann cells in the mutant expressed P0 and MBP with a temporal expression profile comparable to the control (unpublished data).

Specification of neurogenin (ngn) 2–dependent sensory progenitor cells requires *β-catenin*

The neuronal subtypes in DRG are generated in two waves (Anderson, 1999). An early wave is dependent on the basic helix-loop-helix (bHLH) transcription factor neurogenin (ngn) 2 and gives rise to tyrosine receptor kinase (trk) B– and trkC-positive neurons (Ma et al., 1999). In contrast, a somewhat later wave of neurogenesis produces mostly trkA-positive neurons and is dependent on the bHLH factor ngn1. Ngn2 is expressed already in migratory crest cells, whereas ngn1 expression is induced only upon cellular association in the forming DRG (Gradwohl et al., 1996; Sommer et al., 1996). To analyze whether the residual neurons found in the mutant at later embryonic stages (Fig. 2) belonged to a particular sensory subtype, we performed in situ hybridization experiments with trkA, trkB, and trkC riboprobes. Although these markers were readily detectable in control DRG at E16.5, none of these neurotrophin receptors appeared to be expressed by the few nf-positive cells present in the mutant (unpublished data). Thus, these data left open whether the early, ngn2-dependent or the later, ngn1-dependent wave of neurogenesis, or both of these, was affected by *wnt1-Cre*–mediated *β-catenin* ablation. To address this issue, we examined the generation of ngn2-expressing sensory progenitor cells in *β-catenin* mutants. Whole mount in situ hybridization analysis performed at E9.5 revealed ngn2 expression in the neural tube, placodes, and neural crest of control embryos (Fig. 3, A and B). In contrast, ngn2-positive cells were virtually absent in the neural crest along the rostro-caudal axis of mutant embryos, whereas neural tube and placodal ngn2 expression was readily detectable (Fig. 3, C and D). Likewise, on transverse sections at E9.0, ngn2-expressing neural crest cells were

found in control, but not mutant, embryos (Fig. 3, E and F). On adjacent sections, sox10-positive neural crest cells were detected emigrating from the neural tube of both control and *β-catenin* mutant embryos, suggesting that neural crest emigration was not generally affected (Fig. 3, G and H). To monitor whether this phenotype reflected a delay in the generation of ngn2-positive cells, the expression of ngn2 was assayed at later developmental stages up to E12. Ngn2 expression in the PNS was also abolished in the mutant at these later stages (Fig. 3, K and L; unpublished data), indicating that the ngn2-dependent sensory sublineage was never generated in the absence of β-catenin activity.

The loss of ngn2-dependent sensory progenitors might be explained by a lineage-specific requirement of *β-catenin* in survival, proliferation, or fate specification of precursor cells. Both in mutant and control embryos at E8.5 and E9, TUNEL labeling, performed to assess cell death, revealed no or only minimal apoptosis in the dorsal trunk neural tube or the early migratory trunk neural crest (Brault et al., 2001; unpublished data). These data rule out the possibility that lineage-specific cell death could lead to the elimination of ngn2-expressing cells in the *β-catenin* mutant. Wnt signaling is involved in cell cycle regulation of neural tube cells and premigratory neural crest (Ikeya et al., 1997; Megason and McMahon, 2002). The drastic reduction of ngn2-positive cell numbers might thus be due to decreased proliferation. Given the apparently normal emigration of sox10-positive cells in mutant embryos (Fig. 3 H), the *β-catenin* mutation would have to specifically affect cell cycle progression of the ngn2-expressing neural crest lineage. A putative lineage-specific change in cell cycle progression might be difficult to detect in vivo. Immunostaining of mutant embryos with anti–phospho-histone H3 as well as BrdU labeling at E9.5 did not provide evidence for reduced proliferation in the dorsal neural tube or premigratory neural crest in the mutant (unpublished data). To further address this issue, we performed cell culture experiments in which the extent of neural crest outgrowth can be assessed. Neural tubes of control and mutant embryos were isolated at E9 and neural crest cells were allowed to emigrate in defined culture conditions permissive for the generation of ngn2-dependent sensory neurons from sox10-positive progenitor cells (Greenwood et al., 1999). In such a system, a reduction of ngn2-positive cell numbers by death or decreased proliferation rates would result in reduced outgrowth of neural crest cells with sensory neuronal potential. 20 h after plating the neural tubes, control and mutant explants formed by emigrating cells were highly similar and mainly composed of cells coexpressing the low-affinity neurotrophin receptor p75 and sox10 (Fig. 4, A–F), which are markers for neural crest stem cells (Stemple and Anderson, 1992; Paratore et al., 2001). Importantly, quantification of the outgrowth area (Huang et al., 1998) did not reveal any significant difference between control and *β-catenin* mutant neural crest explants (Fig. 4 G), demonstrating that emigration of mutant neural crest cells was normal, and the size of mutant and control explants was similar. Taken together, our data suggest that the early loss of ngn2-expressing cells was not due to a lineage-specific elimination of premigratory or migratory neural crest cells by increased cell death or decreased proliferation.

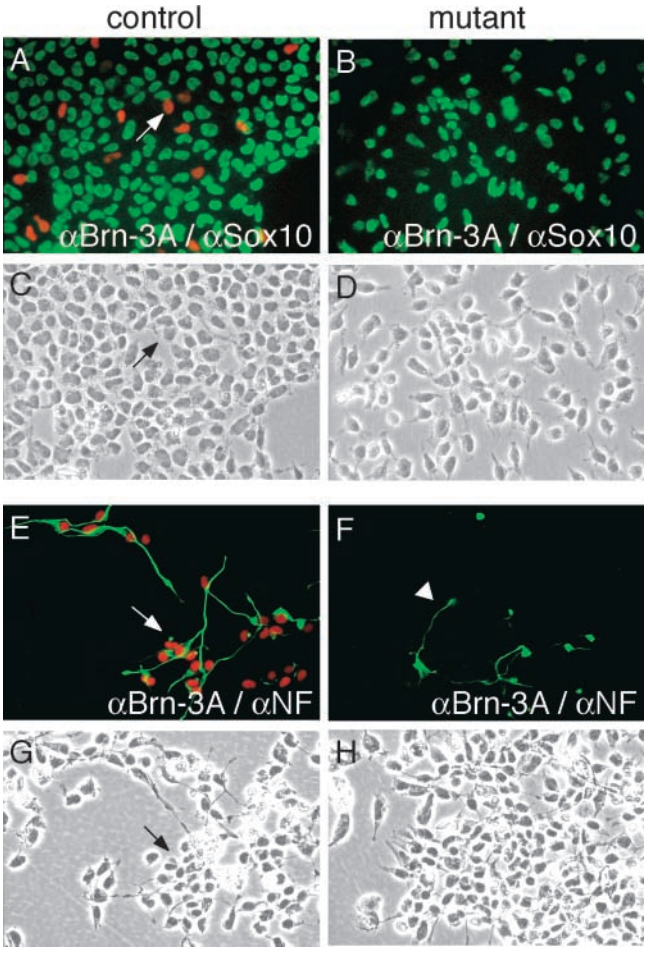


Figure 5. **Mutant neural crest cells are unable to generate sensory neurons.** Neural crest explants from control and mutant mice were prepared as described in the legend to Fig. 4. The cells were allowed to differentiate and were fixed after 36 h (A–D) or 48 h (E–H) in culture. The cultures were immunolabeled using anti–Brn-3A antibody (visualized by Cy3 fluorescence) (A, B, E, and F) and double stained either with anti-sox10 antibody (visualized by FITC fluorescence) (A and B) or anti–nf 160 (NF) (visualized by FITC fluorescence) (E and F). Sensory neuron precursors, defined by the expression of Brn-3A (A, arrow), and sensory neurons, defined by coexpression of Brn-3A and NF (E, arrow), were completely absent in mutant explants (B and F). Note that a few Brn-3A–negative nonsensory neurons were found in mutant explants (F, arrowhead). (C, D, G, and H) Corresponding phase contrast pictures.

A potential role of *β-catenin* in lineage specification might be reflected by altered gene expression in mutant cells. Among various markers tested, we found expression of wnt1 to be transiently maintained in emigrating neural crest cells, whereas usually wnt1 is rapidly down-regulated in wild-type neural crest cells as they emerge from the neural tube (Fig. 3, I and J). At later stages, this ectopic wnt1 expression was abolished (unpublished data). These data might be interpreted to mean that neural crest cells that would normally express ngn2 are unable to do so in *β-catenin* mutants and aberrantly express wnt1. To further support the hypothesis that *β-catenin* is involved in the specification of the early sensory neuronal lineage, a cell culture system was applied that allows monitoring of neuronal fate acquisition by neural crest cells on the cellular level. Neural crest stem cells

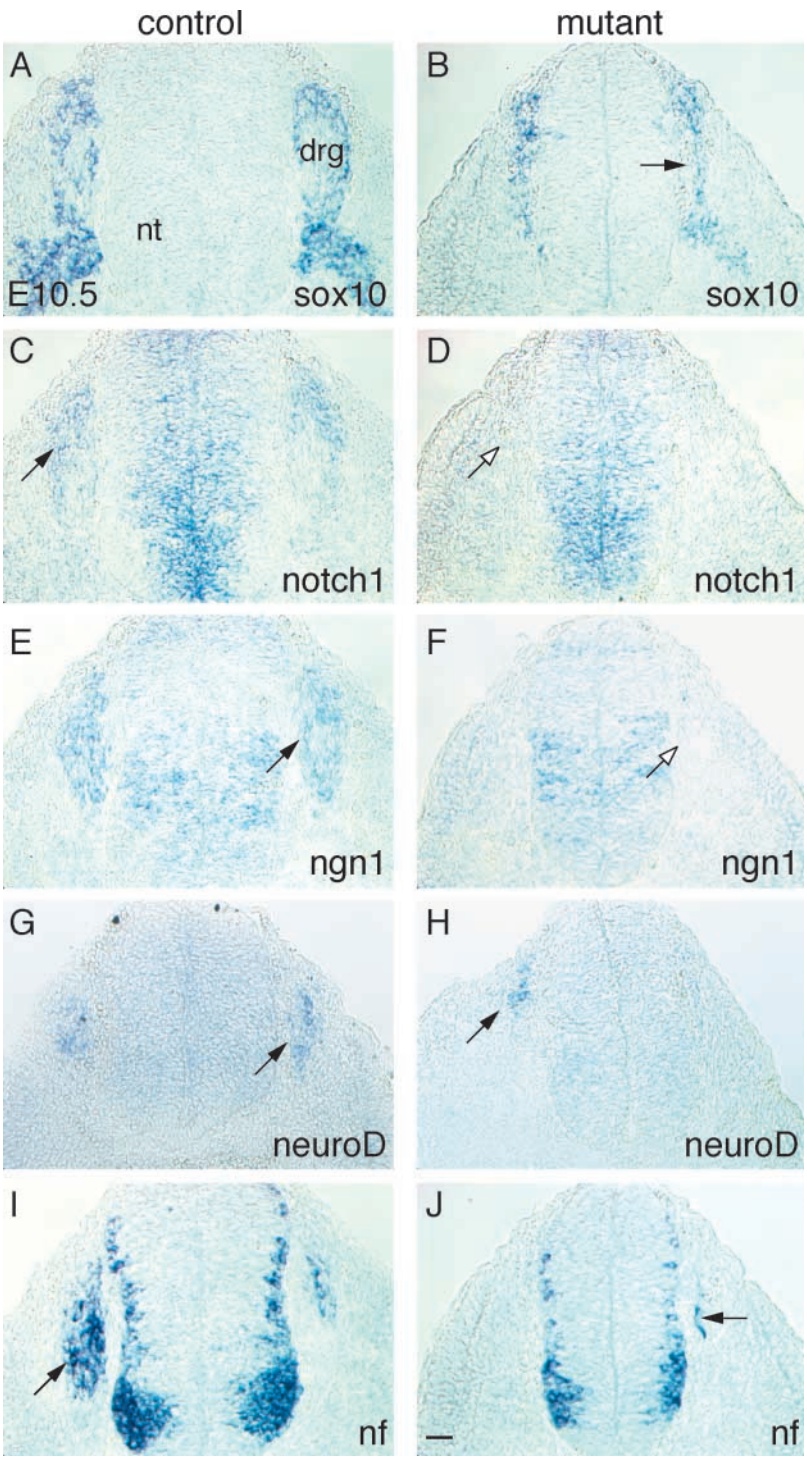


Figure 6. **Failure of DRG formation from sox10-expressing neural crest–derived progenitor cells.** Sox10-expressing cells were present in condensing DRG of control embryos (A) and lateral to the neural tube (nt) of mutant embryos (B, arrow) at E10.5. Near-adjacent sections hybridized with Notch1 riboprobes demonstrate lack of notch1 expression in sox10-positive progenitors of mutant embryos (D, open arrow), whereas Notch1 is expressed in progenitors of control embryos (C, arrow). Furthermore, ngn1-expressing cells were virtually absent lateral to the neural tube of mutant embryos (F, open arrow), whereas extensive ngn1 expression was found in the forming DRG of control embryos (E, arrow). In contrast to control embryos (G and I, arrows), only a few neuroD- and nf-expressing cells were present in mutant embryos (H and J, arrows). Bar, 20 μm.

were cultured in conditions that promote the generation of ngn2-dependent (but not ngn1-dependent) sensory neurons, as described before (Greenwood et al., 1999). As mentioned above, mutant cells emigrated normally from the neural tube in these conditions (Fig. 4). Upon continued culturing of such neural crest explants, many cells in control cultures adopted a sensory neuronal fate characterized by down-regulation of sox10 expression and induced expression of the POU domain transcription factor Brn-3A (Gerrero et al., 1993; Greenwood et al., 1999) (Fig. 5 A). In contrast, mutant neural crest cells maintained their sox10 expression and were unable to generate any Brn-3A–positive sensory neuronal precursors (Fig. 5 B). At a later time point, control explants had generated multiple sensory neurons, identified by coexpression of Brn-3A and NF, whereas in mutant cultures, we never observed sensory neuron formation (Fig. 5, E–H). Occasionally, NF-positive cells were found in the mutant cultures (Fig. 5 F, arrowhead). These were, however, Brn-3A negative, suggesting that some nonsensory neurons formed from mutant neural crest. Thus, signaling mediated by β-catenin is required for the specification of ngn2-dependent sensory neurons.

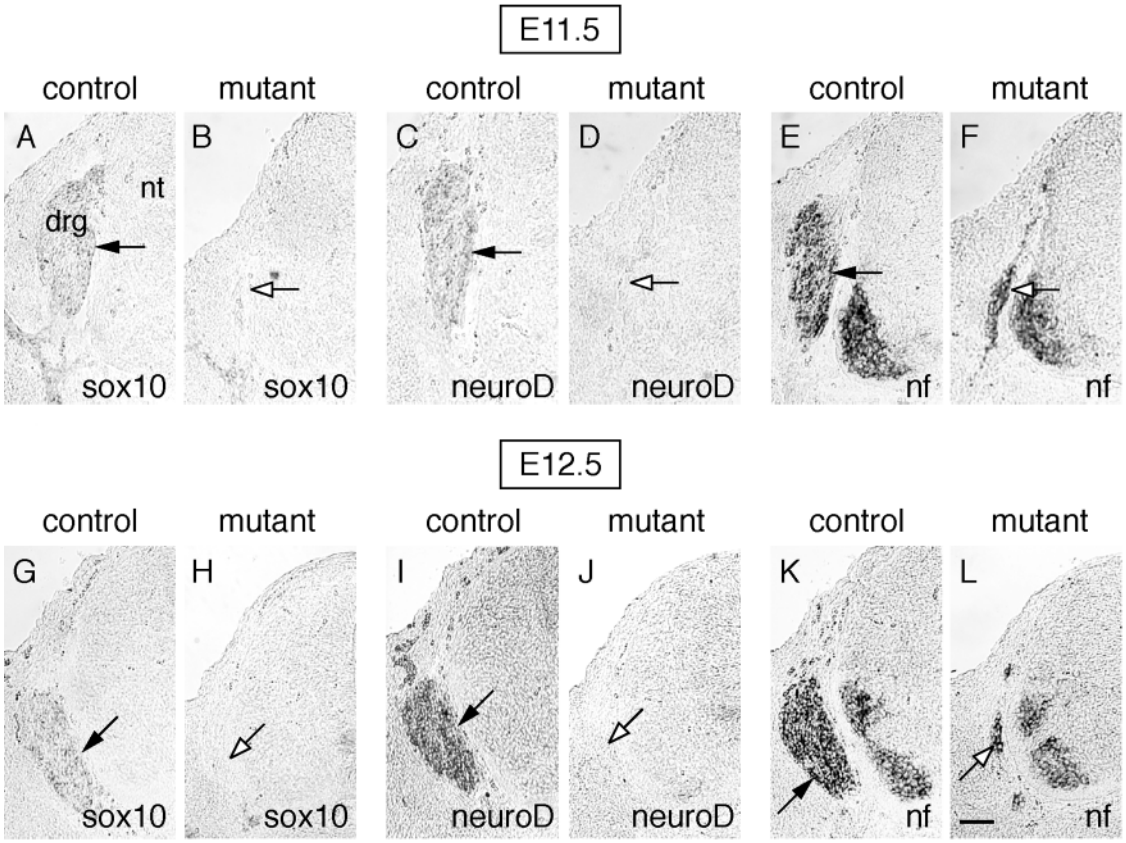


Figure 7. **Absence of de novo neurogenesis in the DRG anlage of mutant embryos at later stages.** At E11.5 (A–F) and E12.5 (G-L), progenitors and glial cells detected by hybridization with a sox10 riboprobe (A and G, arrows), as well as neurons and their precursors detected by expression of nf (E and K, arrows) and neuroD (C and I, arrows) mRNA, respectively, constituted the DRG in control embryos. In contrast, sox10- (B and H, open arrows) and neuroD- (D and J, open arrows) positive cells were completely missing in the mutant. Moreover, only a few nf-expressing neurons were present in mutant embryos (F and L, open arrows). Bar, 50 μm.

Impaired de novo neurogenesis in the DRG anlage

In the next set of experiments, we investigated whether *β-catenin* might play a role in the generation of the second, *ngn1*-dependent wave of sensory neurons. To this end, in situ hybridization experiments were performed at stages when neural crest–derived cells in aggregating DRG up-regulate ngn1 expression and contribute to extensive de novo neurogenesis. Sox10 and Notch1 were used as markers for undifferentiated progenitor cells, whereas ngn1, neuroD, and nf were used to mark the neuronal lineage. At E10.5, sox10-positive neural crest cells were found concomitantly with many ngn1-expressing progenitors in control DRG (Fig. 6, A and E). In control animals, neurogenesis was apparent by the expression of neuroD, a bHLH factor acting downstream of ngns (Ma et al., 1996), and by the presence of differentiated nf-positive neurons (Fig. 6, G and I). In the mutant, however, sox10-expressing cells were present in the area in which ganglia formation occurs in the wild type, but these cells did not appear to form proper ganglia (Fig. 6 B). Moreover, mutant neural crest cells did not express Notch1, unlike their control counterparts (Fig. 6, C and D). Neurogenesis was drastically reduced in the mutant, with virtually no detectable ngn1-expressing cells and little neuroD expression (Fig. 6, F and H). Consequently, few differentiated neurons marked by nf were detectable in the mutant in the region of normal DRG formation (Fig. 6 J).

Sensory neurogenesis was even more affected at E11.5 and E12.5. In control animals at these stages, sox10 expression in progenitors and presumptive satellite glia outlined the DRG, in which multiple neuroD-positive neuronal precursors coexisted with differentiated neurons (Fig. 7). In contrast, neuroD-expressing cells were missing in the mutant (Fig. 7, D and J), indicating that no new neurons were added to the few nf-positive cells that had been born at earlier stages (Fig. 7, F and L). Intriguingly, sox10 expression was also absent lateral to the neural tube and around the few sensory neurons of *β-catenin* mutant embryos (Fig. 7, B and H). Thus, progenitor cells that had been manifest at earlier stages in the area of normal DRG formation (Fig. 6) had disappeared in the mutant by E11.5.

In vivo fate mapping of neural crest cells in control and mutant embryos

Cell death might be an explanation for the loss of neural crest cells that fail to form DRG in mutant embryos. To address this, staining for activated caspase3 or TUNEL assays were performed at E10.5, E11.5, and E12.5 (Fig. 8 C; unpublished data). However, we were unable to detect increased cell death in the mutant embryos in locations where DRG cells normally aggregate in the wild type. Apart from cell death, there are alternative explanations for the disap-

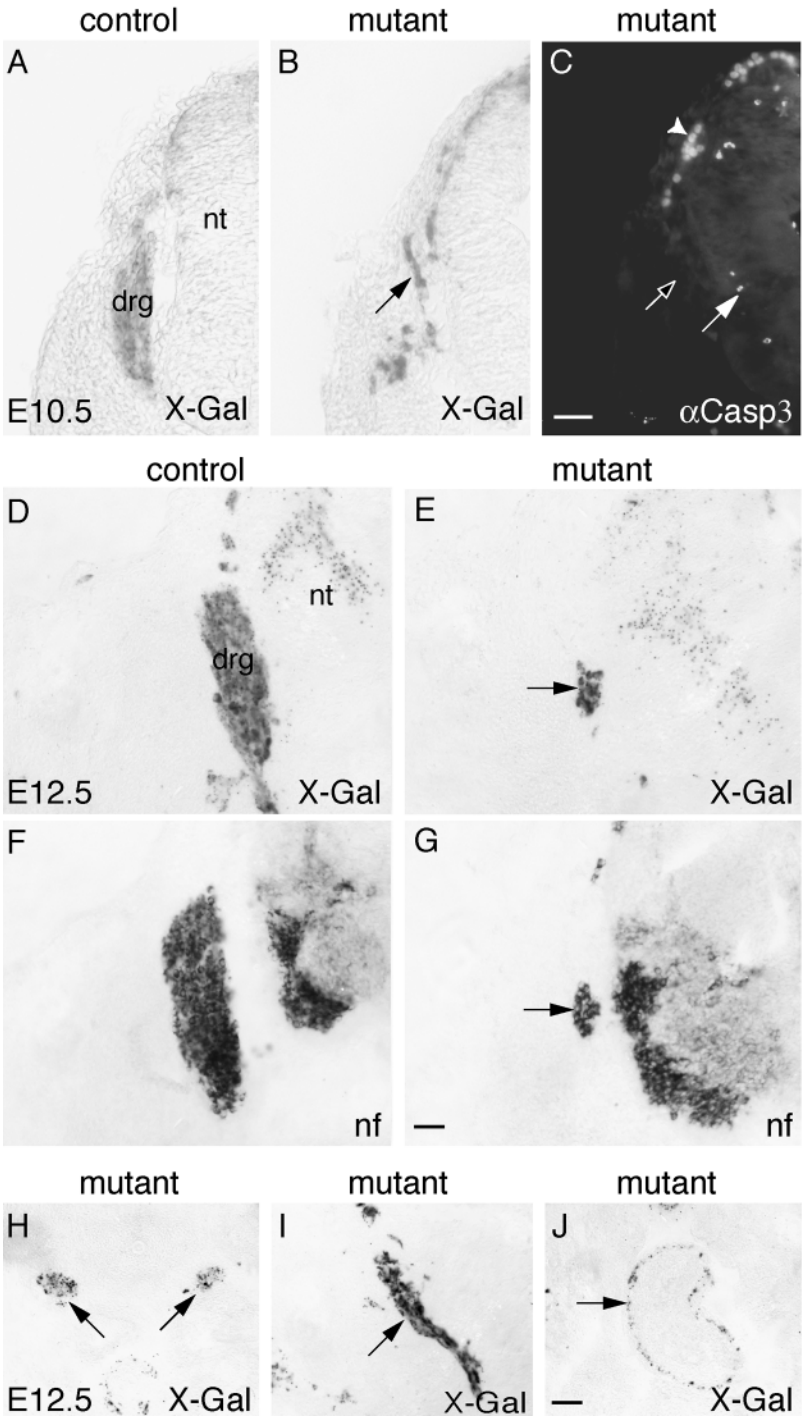


Figure 8. **Survival and in vivo fate mapping of mutant cells.** Control embryos carrying *wnt1-Cre* and the *R26R* allele displayed β-galactosidase activity in the developing DRG at E10.5 (A) and E12.5 (D). At E12.5, the control DRG was outlined by nf expression (F). Cre-expressing neural crest cells and their derivatives were also detectable by β-galactosidase expression in mutant embryos carrying *wnt1-Cre* and the *R26R* allele. At E10.5, mutant cells were localized lateral to the neural tube without forming proper DRG (B). No increased cell death was found in this area, as shown by immunostaining for the activated form of caspase3 (Casp3) (C, open arrow). B and C are adjacent sections. Note the Casp3-positive cells within the neural tube (arrow) and autofluorescent blood cells present at the dorsal margins of the embryo (arrowhead). At E12.5, neural crest–derived mutant cells expressing β-galactosidase (E, arrow) were confined to the domain of a few nf-positive cells present in the mutant (G, arrow). E and G represent adjacent sections. Mutant cells were able to normally colonize other neural crest–derived structures, such as sympathetic ganglia (H, arrows), peripheral nerves (I, arrow), and the enteric nervous system (J, arrow). Bars: (A–C) 50 μm; (D–J) 100 μm.

pearance of progenitor cells in the area of normal DRG formation. Mutant neural crest–derived cells might be able to localize to the site of normal DRG formation but might adopt an aberrant fate not detected by the neural markers tested so far. To investigate this issue, we performed in vivo fate mapping experiments using the ROSA26 Cre reporter allele *(R26R)* (Soriano, 1999). Upon Cre-mediated recombination, β-galactosidase is expressed from this allele in Cre-expressing cells and their progeny. Thus, the fate of control and *β-catenin* mutant neural crest cells can be followed in compound transgenic animals expressing *Cre* from the *wnt1* promoter. In animals wild type for *β-catenin*, β-galactosidase expression delineated all neural crest–derived structures at E10.5 and E12.5 (Fig. 8, A and D; Jiang et al., 2000). In *β-catenin* mutant embryos at E10.5, mutant cells expressing β-galactosidase were localized in streams lateral to the neural tube without forming proper DRG (Fig. 8 B). This expression pattern correlated with domains of sox10 expression on adjacent sections (unpublished data; Fig. 6). Most importantly, at E12.5, comparison of β-galactosidase and nf expression revealed that the only neural crest–derived cells that localized lateral to the neural tube were the few sensory neurons present in *β-catenin* mutants (Fig. 8, E and G). In contrast, similar to control cells, mutant β-galactosidase–

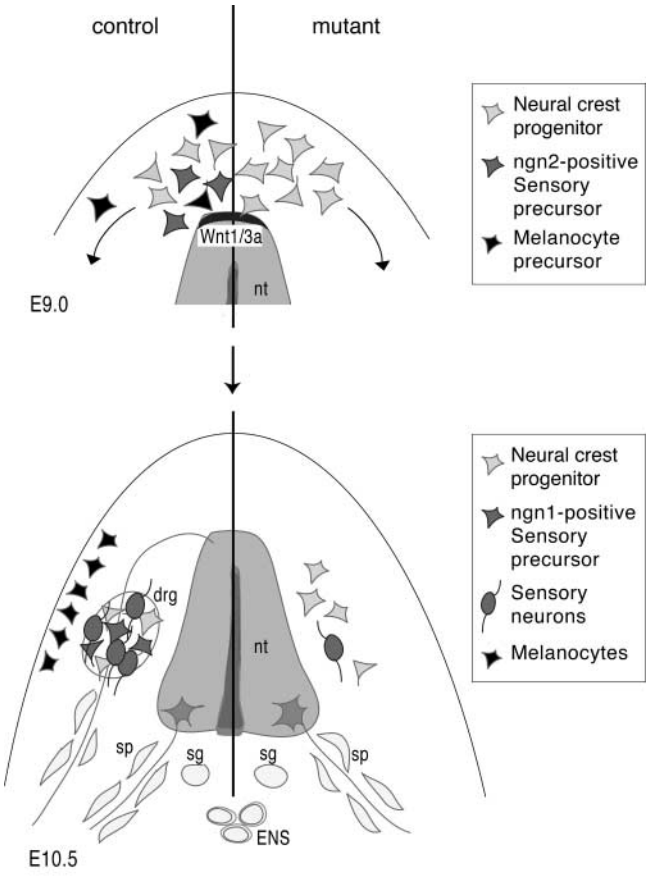


Figure 9. **Lineage-specific requirement of *β-catenin* in neural crest development.** (E9.0, control) Emigrating neural crest appears to be heterogeneous, consisting of multipotent neural crest progenitor cells, early sensory precursors (marked by ngn2 expression), and possibly melanoblasts. (E9.0, *β-catenin* mutant) Neural crest fails to generate sensory precursors and melanoblasts. The specification of sensory and melanocytic fates in premigratory or early migratory neural crest might depend on signaling by wnt1/wnt3a, which are expressed in the dorsal neural tube (nt) at the stage of crest emigration. (E10.5, control) Neural crest–derived progenitor cells aggregate in DRG and produce ngn1-dependent sensory precursors. (E10.5, mutant) Progenitors lateral to the neural tube fail to aggregate and to form proper DRG; virtually no ngn1-expressing sensory precursors and only very few differentiated sensory neurons are detectable. This might point to a role of β-catenin in mediating cell–cell interactions possibly involved in sensory neurogenesis, although other β-catenin functions cannot be excluded. Other crest derivatives such as sympathetic ganglia (sg), the enteric nervous system (ENS), and Schwann cell precursors along peripheral nerves (sp) form independently of β-catenin activity.

positive cells localized to sympathetic ganglia (Fig. 8 H), nerves (Fig. 8 I), and the enteric nervous system (Fig. 8 J). Hence, in vivo fate mapping did not reveal aberrant generation of nonneural cells from mutant progenitors in the area of normal DRG formation. Rather, mutant cells gave rise to multiple structures of the PNS while they failed to form proper DRG.

Discussion

In this study, we identify β-catenin as a crucial signal in neural crest development. Conditional *β-catenin* gene ablation in the dorsal neural tube and neural crest stem cells prevents the generation of melanoblasts and ngn2-dependent sensory neurons, presumably reflecting a role of *β-catenin* in premigratory crest or at early stages of emigration (Fig. 9). Moreover, *β-catenin* mutant neural crest cells fail to aggregate in DRG and to produce ngn1-dependent sensory neurons, whereas other neural crest derivatives, such as the enteric nervous system and sympathetic ganglia, form normally (Fig. 9). Analysis of mutant and control embryos, together with in vivo fate mapping and cell culture experiments, indicates a lineage-specific requirement of *β-catenin* for the specification of both melanocytes and sensory neuronal lineages from neural crest cells.

Role of β-catenin in melanocyte formation

Apart from its role in neural crest induction and expansion at early developmental stages (Ikeya et al., 1997; Saint-Jeannet et al., 1997; LaBonne and Bronner-Fraser, 1998), Wnt signaling has also been directly associated with the formation of the melanocyte lineage from neural crest. Both in avian cell cultures and in zebrafish in vivo, activation of the Wnt signaling pathway in neural crest cells promoted the formation of pigment cells (Dorsky et al., 1998; Jin et al., 2001). In agreement with a role of Wnt signaling in melanocyte formation, ablation of *β-catenin* in mouse neural crest cells not only leads to the loss of the melanocyte differentiation marker trp2 but also of mitf, a bHLH zipper transcription factor regulating melanoblast specification (Opdecamp et al., 1997; Yasumoto et al., 1997). This finding might reflect a direct relationship between β-catenin–dependent signaling and induction of *Mitf* gene expression, as the *Mitf* promoter harbors a binding site for a β-catenin–containing transcription factor complex and can be activated by Wnt (Dorsky et al., 2000b; Takeda et al., 2000). It has been reported that in compound mutant mice lacking both *wnt1* and *wnt3a*, late-emigrating neural crest lineages are missing because of a general reduction of the neural crest cell population, which would primarily affect later- rather than earlier-forming neural crest derivatives (Ikeya et al., 1997). This is an unlikely explanation for the lack of melanocytes in *β-catenin* mutant embryos because, unlike in chicken, melanocyte-forming neural crest cells in mouse embryos migrate along the dorsolateral pathway throughout the period of migration, including at early stages (Serbedzija et al., 1990). Taken together, the data indicate that β-catenin plays a role in specifying the melanocyte lineage from neural crest cells, most likely due to its function in mediating Wnt signaling (Fig. 9). Specification and early differentiation of melanocytes occurs in the so-called migration staging area, a space rich in extracellular matrix that is localized lateral to the dorsal neural tube (Wehrle-Haller and Weston, 1997; Dorsky et al., 2000a). In some, but not all, mutant embryos analyzed, we observed increased apoptosis in this area at E10.5 (but not at other stages) (unpublished data), consistent with the idea that at least some cells that fail to be specified as melanocytes are subsequently eliminated by cell death. If so, this effect might be lineage specific, because cell death in other neural crest derivatives, such as in the DRG anlage, was apparently not increased in the mutant.

Early specification of ngn2-dependent sensory neuronal cells by β-catenin

Neurogenic and melanogenic lineages segregate during the earliest stages of neural crest development (Henion and Weston, 1997). Furthermore, the promotion of melanocyte formation by Wnt signaling has been reported to occur at the expense of neural lineages (Dorsky et al., 1998; Jin et al., 2001). However, our analysis of neural structures in *β-catenin* mutant embryos showed that, similar to the melanocyte lineage, particular neural structures are reduced in size rather than increased. Thus, our data do not support a model in which β-catenin regulates a fate switch between neurogenic and melanogenic lineages. The discrepancy in results (Dorsky et al., 1998; Jin et al., 2001; this study) might be explained by distinct timing of interference with the Wnt signaling pathway, differential roles of Wnt and β-catenin, and species differences.

Although the reduction of peripheral nerves observed in *β-catenin* mutants is presumably secondary to the absence of sensory neurons, the lack of ngn2-dependent sensory neurons and their progenitors most likely reflects a primary role of β-catenin in the formation of this lineage. Normally, ngn2 expression is detectable in a subset of sox10-positive neural crest cells as they emerge from the neural tube (Sommer et al., 1996; Lo et al., 2002). In *β-catenin* mutants, sox10-positive cells emigrate in vivo but ngn2 expression is absent or restricted to a very few neural crest cells (Fig. 9). Similarly, sox10-positive neural crest cells emigrate in neural tube explants in vitro but these cells are unable to adopt a sensory neuronal fate, even in conditions promoting ngn2-dependent sensory neurogenesis. These data indicate that neural crest cells that would normally express ngn2 emigrate, but fail to acquire a sensory neuronal fate in the absence of *β-catenin*. Alternatively, ngn2-positive cells or their progenitors might be selectively eliminated in the mutant neural tube before or at emigration. However, at E8.5 and E9, we did not observe increased cell death in premigratory and migratory neural crest in *β-catenin* mutant embryos as compared with control embryos (this study; Brault et al., 2001). Moreover, the normal outgrowth of mutant neural crest explants in culture also speaks against a lineage-specific elimination of ngn2-positive cells.

In vivo, the increased expression of wnt1 in neural crest cells lacking *β-catenin* suggests that wnt1 expression is involved in a negative regulatory feedback loop with β-catenin. Moreover, this finding also allows the assumption that β-catenin controls the early specification of ngn2-dependent sensory neurons by mediating Wnt signaling rather than cadherin-dependent cell adhesion. Thus, β-catenin, presumably as a component of the Wnt signal transduction pathway, can be added to the list of signals, such as TGFβ family members, Notch, and NRG, that regulate cell fates in neural crest development (Anderson et al., 1997; Sommer, 2001). It remains to be determined whether this function is exerted by direct regulation of gene expression of the *ngn*s that encode bHLH factors specifying sensory neuron identity (Ma et al., 1999; Perez et al., 1999).

The absence of neural crest cells marked by ngn2 not only points to a role of *β-catenin* at earliest stages of neural crest development but also suggests that the ngn2-positive lineage might segregate from other neural crest lineages already before or shortly after delamination from the neural tube (Fig. 9). In agreement with this, in vivo fate mapping by dye injection revealed that most (but not all) neural crest cells were restricted to generate either sensory or autonomic neurons, but not both neuronal subtypes (Frank and Sanes, 1991; Fraser and Bronner-Fraser, 1991). Similarly, sensory precursors in neural crest explants turned out to be determined, unable to respond to factors inducing autonomic neurogenesis (Greenwood et al., 1999). Moreover, lineage-tracing experiments have recently demonstrated that ngn2-expressing cells preferentially contribute to sensory rather than autonomic ganglia (Zirlinger et al., 2002). Given the early function of β-catenin in sensory neurogenesis and given the expression of Wnts before neural crest emigration, the question arises of how undetermined cells with competence to generate derivatives other than sensory neurons are being maintained in the neural crest. β-Catenin signaling can be modulated by the activity of other signaling molecules, such as components of the Notch pathway and TGFβ family members (Hecht and Kemler, 2000; De Strooper and Annaert, 2001). Thus, the biological response of a neural crest cell to β-catenin might depend on its cellular context, which determines the signal combination and concentration as well as the timing of signaling that the cell is exposed to.

β-Catenin and neurogenesis during peripheral ganglia formation

Although the number of sensory neurons is drastically reduced in the DRG anlagen of *β-catenin* mutant embryos, a few sensory neurons are born at early developmental stages. From E11.5 onwards, however, sensory neurogenesis, as assayed by neuroD expression, was completely abolished in the mutant. The early generation of the residual neurons might suggest that they are derived from the early, ngn2-dependent rather than from the later, ngn1-dependent wave of neurogenesis. This issue was, however, difficult to assess because of the low or absent expression of markers that would distinguish sensory neuronal sublineages, such as ngn1 and ngn2 and trkA, B, and C (Anderson, 1999). The absence of neurotrophin receptor expression in the few neurons present in the mutant suggests that proper differentiation of sensory neurons either requires β-catenin activity in a cell-autonomous manner or depends on cellular interactions with neural cells missing in the mutant. In any case, however, our data clearly demonstrate a requirement of *β-catenin* in the formation of both ngn2- as well as ngn1-dependent sensory neurons (Fig. 9).

It is unclear whether the virtual absence of ngn1 expression in the mutant reflects a role of β-catenin in Wnt signaling or in mediating cadherin-dependent cellular adhesion. Interference with Wnt signaling in the neural crest does not just lead to a phenocopy of *β-catenin* mutants (Ikeya et al., 1997; Brault et al., 2001), suggesting Wnt-independent roles of *β-catenin* in neural crest development. However, the differences in phenotypes could reflect different stages at which the Wnt signaling pathway has been perturbed or could be due to the fact that *β-catenin* gene deletion affects signaling by different Wnt family members. Hence, we can-

not exclude, as proposed above for the ngn2-dependent early wave of sensory neurons, that later-forming sensory lineages would also be specified by Wnt signaling. Alternatively, interference with cadherin-dependent cell adhesion rather than Wnt signaling might lead to the absence of DRG formation in *β-catenin* mutants. In support of this idea, cell–cell adhesion is defective in *β-catenin* mutant embryos (Haegel et al., 1995; Brault et al., 2001). Moreover, there is circumstantial evidence that cellular interactions might be involved in sensory neurogenesis. Normally, ngn1 is induced as neural crest–derived cells aggregate to form DRG (Sommer et al., 1996), but it is not known whether there is a causal relationship between cellular association and ngn1 expression. Short-range cell–cell interactions, termed community effects, influence the response of multipotent progenitor cells to extracellular factors, thereby promoting neurogenesis at the expense of a nonneural fate (Hagedorn et al., 1999, 2000a; Paratore et al., 2001). Furthermore, it has been suggested that early differentiating neurons might interact with DRG progenitor cells and serve as a "scaffold" for later-born sensory neurons (Anderson, 1999). According to this idea, the strong reduction of early-born sensory neurons in *β-catenin* mutant embryos would impede ngn1-dependent sensory neurogenesis. Analysis of sox10 expression reveals that *β-catenin* gene ablation does not only affect the induction of neuronal traits in undifferentiated progenitor cells. Rather, ganglionic structures delineated by sox10-positive cells are not even formed, suggesting that undifferentiated progenitor cells fail to aggregate in DRG at any developmental stage (Fig. 9).

The absence of proper ganglia expressing sox10 is not just due to aberrant down-regulation of this marker, as demonstrated by in vivo fate mapping of mutant cells. *β-catenin* mutant neural crest cells expressing β-galactosidase from the recombined *R26R* allele emigrate normally, are then found for a transient period lateral to the neural tube without forming overt DRG, and are later confined to the few sensory neurons present in the mutant. Thus, mutant cells do not adopt an alternative, nonneural fate in the area of normal DRG formation. Moreover, the phenotype is unlikely to be explained by mutant cells that would aggregate but then die, as we found no evidence for increased cell death in the mutant DRG anlage at any stage analyzed. Hence, the most easily conceivable interpretation of our data is that mutant neural crest cells fail to aggregate in DRG and to generate sensory neurons and satellite glia, and instead populate other neural crest–derived structures. However, we did not observe an increase in nonsensory neural crest derivatives, suggesting that secondary mechanisms might regulate the generation of correct cell numbers in these tissues. Whether our data reflect the existence of migratory progenitors that in the wild type are able to generate both sensory as well as other neural crest–derived cell types remains to be addressed. In any case, however, the results are consistent with the hypothesis that cell–cell interactions are required for the promotion of neural fates in developing DRG. Signaling by Notch1, which is absent in the DRG anlage of *β-catenin* mutants, might be involved in this process. Moreover, such cellular interactions are likely to be mediated by N-cadherin–containing adhesion complexes, as progenitor cells aggregating in DRG express N-cadherin (unpublished data; Pla et al., 2001). Further experiments are required to elucidate the role of cell–cell interactions in promoting sensory neurogenesis.

Materials and methods

Mating scheme and genotyping

Breeding of mice and genotyping were performed as described in Brault et al. (2001). Embryos heterozygous for the *β-catenin* floxed and floxdel alleles and carrying the *wnt1-Cre* transgene were referred to as mutant embryos, whereas littermates that inherited the incomplete combination of the above alleles served as control animals (Brault et al., 2001).

In situ staining procedures

Nonradioactive in situ hybridization with digoxigenin-labeled riboprobes was performed on cryosections as previously described (Paratore et al., 1999). NBT/BCIP (Roche Diagnostics) were used as chromogens to visualize hybridization signals. Antisense riboprobes were labeled with digoxigenin according to the manufacturer's instructions (Roche Diagnostics). TUNEL staining and BrdU assays were performed according to the manufacturer's instructions (Roche Diagnostics). Immunostainings for the active form of caspase3 were performed on cryosections fixed for 15 min in 4% paraformaldehyde in PBS at RT. Sections were treated with blocking buffer (10% goat serum, 0.3% Triton, 0.1% BSA in PBS) for 30–60 min at RT before incubation with polyclonal rabbit anti-caspase3 antibody (1:100 dilution; BD Biosciences) for 2 h at RT. For anti–phospho-histone H3 stainings, the cryosections were fixed for 10 min in acetone at −20°C and incubated with a polyclonal rabbit antibody (diluted 1:500; Upstate Biotechnology) overnight at 4°C. Stainings were visualized by incubation for 1 h at RT using a Cy3-conjugated goat anti–rabbit IgG antibody (1:200 dilution; Jackson ImmunoResearch Laboratories). X-Gal staining on cryosections was done as previously described (Lickert et al., 2002).

Cell culture

Neural crest cultures in conditions permissive for sensory neurogenesis were essentially prepared as previously reported (Greenwood et al., 1999). After 20 h of neural crest emigration, the neural tubes were removed from the cultures and used for genotyping by PCR (Sommer et al., 1995). To quantify the extent of the neural crest outgrowth, digital images of the explant cultures at 20 h were acquired by video microscopy using 5× magnification. NIH image 1.62 software was used to measure the size of the explants. To this end, the outgrowth area (mm^2) was divided by the perimeter (mm) of the explant. This normalized number (in mm) is referred to as the migration index and provides an estimate of the total outgrowth of a neural crest explant (Huang et al., 1998).

Immunocytochemistry

After fixing the cells with 3.7% formaldehyde in PBS for 10 min, the cells were treated for 10 min at RT with blocking buffer (10% goat serum, 0.3% Triton X-100, 0.1% BSA in PBS). Neural crest cells were labeled with rabbit anti–mouse p75 (1:300 dilution; Chemicon International) for 1 h at RT and with monoclonal anti-sox10 antibody (1:10 dilution; Paratore et al., 2001) for 2 h at RT. Polyclonal rabbit anti–Brn-3A antibody (Fedtsova and Turner, 1995) and monoclonal anti-NF160 antibody NN18 (IgG) (Sigma-Aldrich) were diluted 1:300 in blocking buffer and stainings were performed at RT for 1 h. Immunostaining was visualized by incubation for 1 h at RT using the following secondary antibodies at 1:200 dilution: Cy3-conjugated goat anti–mouse IgG; Cy3-conjugated goat anti–rabbit IgG; FITC-coupled donkey anti–rabbit IgG (all from Jackson ImmunoResearch Laboratories); and FITC-coupled horse anti–mouse IgG (Vector Laboratories).

We thank Uli Müller for helpful discussions and Ned Mantei for critical reading of the manuscript. We are grateful to Andrew McMahon (Harvard University, Cambridge, MA) and Philippe Soriano (Fred Hutchinson Cancer Research Center, Seattle, WA) for providing transgenic animals used in this study, and we acknowledge the following scientists for riboprobes: Hauke Werner (ZMBH, Heidelberg, Germany) (erbB3); Alexander Gow (Wayne State University, Detroit, MI) (mbp); Michael Wegner (University of Erlangen, Erlangen, Germany) (mitf and sox10); Jackie Lee (University of Colorado, Boulder, CO) (neuroD); Monika Oblinger (Chicago Medical School, North Chicago, IL) (nf); Freddy Radtke (Ludwig Institute, Lausanne, Switzerland) (notch1); Silvia Arber (University of Basel, Basel, Switzerland) (trkA, B, and C); Bernhard Wehrle-Haller (University of Geneva, Geneva,

Switzerland) (trp2); and Roel Nusse (Stanford University, Stanford, CA) (wnt1). We thank Michael Wegner and Eric Turner (University of California, San Diego, CA) for antibodies.

This work was supported by grants of the Swiss National Science Foundation (to L. Sommer and U. Suter) and by the National Center of Competence in Research "Neural Plasticity and Repair."

Submitted: 9 September 2002
Revised: 29 October 2002
Accepted: 30 October 2002

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Instructive role of Wnt/ β-Catenin in sensory fate specification in neural crest stem cells

Science **303** (5660): 1020-1023; 2004

My contribution to this work:

- Discovery of the in vivo phenotype and development of the hypothesis of Wnt acting as an instructive factor for sensory fate specification, together with L. Sommer.
- Collection of first in vivo data, which in the end led to Fig. 1E-H, Fig. 2Q-V, Fig. S2E-H
- Proofreading of the manuscript

cells, in contrast to control cells (Fig. 4E). The expression pattern of IκBβ correlated well with that of Foxj1, diminishing particularly in response to anti-CD3 or IL-2 stimulation (fig. S1B). In addition, transduction of primary Th cells by Foxj1 increased IκBβ expression (fig. S1C), and Foxj1 could transactivate the IκBβ promoter (fig. S1D). Finally, Foxj1$^{-/-}$ T cells contained diminished levels of IκBβ mRNA (Fig. 4F). These findings strongly suggest that Foxj1 antagonizes NF-κB activity at least in part by inducing and/or maintaining IκB activity. As a consequence of this, deficiency in Foxj1 leads to spontaneous NF-κB activation and subsequent immune dysregulation.

We suggest that Foxj1 regulates early Th activation, enforcing T cell quiescence by regulating NF-κB activity, in part via IκB (fig. S6). However, recent studies suggest that NF-κB may preferentially play a role in later, postcommitment phases of Th1 development and proliferation (*14*), suggesting that Foxj1 may also enforce quiescence by modulating the activity of another class(es) of transcriptional regulators and/or may have as-yet undefined direct effects on T cell differentiation genes, such as LKLF (*18*) or Tob (*19*). Regardless, the present findings are consistent with prior observations demonstrating dysregulated NF-κB activity in both human (*20*) and murine (*21*, *22*) lupus and inflammatory phenotypes of animals deficient in IκB activity (*23*) and furthermore are consistent with the significantly reduced expression of Foxj1 in murine lupus Th cells (fig. S1). Thus, studies to define further the target genes regulated by Foxj1 as well as other Fox transcription factors will undoubtedly shed insight into the regulation of Th differentiation and immune tolerance.

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24. We are grateful to T. Kitamura for the pMX/PlatE retroviral system; R. Schreiber for the use of the ABI PRISM (Applied Biosystems, Foster City, CA) sequence detection system; M. Oukka and L. Glimcher for the NF-κB-luc construct and the 293-T cell line; M. R. Marshall for technical assistance; and J. Green, K. Murphy, A. Shaw, and W. Yokoyama for critical commentary during the preparation of the manuscript. This work was supported in part by the Siteman Cancer, Diabetes Research and Training, and the Digestive Diseases Research Core (DK52574) Centers of the Washington University School of Medicine, as well as grants from NIH (AI01803 and AI057471 to S.L.P. and HL56244 and HL63988 to S.L.B.), the Lupus Research Institute, and the Broad Medical Research Foundation. S.L.P. is supported in part by an Arthritis Investigator award from the Arthritis Foundation. S.L.B. is a recipient of a Career Investigator award from the American Lung Association.

Supporting Online Material
www.sciencemag.org/cgi/content/full/303/5660/1017/DC1
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21 November 2003; accepted 19 December 2003

Instructive Role of Wnt/β-Catenin in Sensory Fate Specification in Neural Crest Stem Cells

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Wnt signaling has recently emerged as a key factor in controlling stem cell expansion. In contrast, we show here that Wnt/β-catenin signal activation in emigrating neural crest stem cells (NCSCs) has little effect on the population size and instead regulates fate decisions. Sustained β-catenin activity in neural crest cells promotes the formation of sensory neural cells in vivo at the expense of virtually all other neural crest derivatives. Moreover, Wnt1 is able to instruct early NCSCs (eNCSCs) to adopt a sensory neuronal fate in a β-catenin–dependent manner. Thus, the role of Wnt/β-catenin in stem cells is cell-type dependent.

Wnt proteins are able to induce proliferation in different types of stem cells (*1*–*3*). In the central nervous system, Wnts act mitogenically on progenitor cells, and activation of β-catenin, a component of the canonical Wnt signaling pathway (*4*), leads to amplification of the neural progenitor pool (*5*, *6*).

The question arises whether canonical Wnt signaling regulates stem cell self-renewal in general or whether this function is cell type–dependent. To address this issue, we analyzed the role of Wnt/β-catenin in NCSCs. Neural crest cells generate most of the vertebrate peripheral nervous system (PNS) and several non-neural derivatives (*7*). Wnt signaling has previously been implicated in early stages of neural crest development, such as neural crest induction and melanocyte formation (*8*–*10*). In NCSCs, specific ablation of the β-*catenin* gene results in lack of melanocytes and sensory neural cells in dorsal root ganglia (DRG) (*11*). NCSCs without β-catenin emigrate and proliferate normally but are unable to acquire a sensory neuronal fate.

These results are consistent with a role of β-catenin signaling in inducing a sensory fate. To address this hypothesis, we used the *cre/loxP* system to generate mice expressing a constitutively active form of β-catenin specifically in neural crest cells (fig. S1) (*12*). We first assessed the developmental potential of control and mutant neural crest cells by performing in vivo fate mapping experiments (*13*). In the control (Fig. 1A), neural crest cells emanating from the anterior neural tube populated the nasofrontal and periocular region, where they become mesenchyme that later generates bones, connective components, and vascular structures of the head (*14*–*16*). Neural crest cells also migrated into branchial arches that contribute to craniofacial skeletal tissue and the major arteries (*14*, *17*, *18*). In the mutant (Fig. 1B), however,

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cranial neural crest–derived cells hardly spread over the forebrain area and were more restricted to ventral head structures. Only a vestigial first branchial arch was present; all other branchial arches were absent. Instead, mutant neural crest cells aggregated in prominent cranial ganglion-like structures dorsal to the normal site of branchial arch formation.

Transverse sections revealed strongly reduced numbers of both neural crest cells in the cardiac outflow tract and neural crest–derived melanocytes (Fig. 1, C to F). In vivo fate mapping also revealed a complete absence of the enteric nervous system (Fig. 1, G and H). Moreover, mutant cells were unable to associate with peripheral axons and, thus, to contribute to the Schwann cell lineage in peripheral nerves (Fig. 1, I and J). The markers sox10 and erbB3 were barely detectable in mutant peripheral nerves (*19*), and cad6-positive cranial nerves were missing (Fig. 2, A and B), confirming the lack of presumptive Schwann cells. Absence of neural crest–derivatives in the mutant is not attributable to cell death, because no increased apoptosis was observed in regions normally composed of neural crest targets (*19*).

Normally, cad6 expression at embryonic day 10.5 (E10.5) is confined to neural structures and absent in mesenchymal neural crest derivatives (Fig. 2, A, C, and E). In the mutant, however, the entire area of β-galactosidase–positive cranial neural crest cells also displayed cad6 expression (Fig. 2, B, D, and F). Within the ectopic domain of cad6 expression, neurofilament (nf) was readily detectable, as were several transcription factors characteristic of the sensory lineage (Fig. 2, H, K, and L) (*19*). Thus, upon sustained activation of β-catenin, sensory neurons are generated in anterior regions of the embryo that are usually devoid of neural derivatives of the neural crest. Similarly, the prominent cranial ganglia (Fig. 1B, arrowheads) contained many cells positive for sensory markers (fig. S2). Evidently, neural crest cells populating these structures have adopted a sensory neural fate.

In the trunk, ngn2 is normally expressed in emigrating neural crest cells and marks cells fated for sensory neural lineages (*20*). Its transient expression in cells aggregating in early DRG is followed by expression of the sensory markers ngn1 and neuroD. Whereas lack of β-catenin abolishes ngn2-expression (*11*), sustained β-catenin activity resulted in increased numbers of ngn2-positive cells, both in the trunk and at ectopic cranial locations (Fig. 2, M to P). Moreover, ngn2 expression was not restricted to the DRG anlage. Rather, ngn2-positive cells appeared to migrate on ventral routes and accumulated lateral to the dorsal aorta at sites of normal sympathetic ganglion formation (Fig. 2, P and R). Ectopic expression of ngn1 and neuroD was also found in these structures (Fig. 2, S and T; fig. S2). In contrast, the autonomic neuronal markers mash1 and ehand were virtually absent in the mutant PNS (Fig. 2, U and V; fig. S2), indicating that sensory neurons were forming at the expense of sympathetic neurons. The data also reveal that, unlike some other neural crest target structures (Fig. 1, C to J), sites of normal sympathetic ganglia development can be populated by sensory neurons.

Thus, β-catenin signaling not only appears to be required for sensory neurogenesis (*11*) but also to promote this fate. To evaluate this further, we investigated the developmental potential of isolated NCSCs expressing stabilized β-catenin. In culture, both control and mutant migratory neural crest expressed the NCSC-markers p75 and Sox10 (Fig. 3, A and B) (*21*). Moreover, migration, proliferation, and cell death was not significantly altered between the explants (Fig. 3, A to F; table S1), indicating that the loss of nonsensory lineages in the mutant is not due to selective mechanisms before or during neural crest emigration. In conditions permissive for sensory neurogenesis, control neural crest cells produced few Brn-3A–positive sensory neurons, and many cells maintained Sox10 expression (Fig. 3; table S1). In contrast, virtually all mutant cells lost Sox10 immunoreactivity and the majority became Brn-3A–positive, demonstrating that β-catenin signaling is able to specify a sensory neuronal fate in most neural crest cells. Because Sox10 has been associated with NCSC maintenance (*22*, *23*), the data also suggest that overexpression of β-catenin promotes the loss of multipotency in NCSCs.

We next investigated whether the effect of activated β-catenin in neural crest cells reflects a role of Wnt signaling. Unlike in control conditions, wild-type NCSCs exposed to Wnt1 efficiently generated Brn-3A–positive sensory neurons (Fig. 4). When we similarly challenged β-catenin–deficient NCSCs with Wnt1, we were unable to detect any sensory neurons (Fig. 4D), in agreement with our previous finding that β-catenin is required for sensory neurogenesis (*11*, *24*). Thus, Wnt1 promotes sensory neurogenesis in a β-catenin–dependent manner.

The effect of Wnt/β-catenin signaling could, in principle, be explained by two distinct mod-

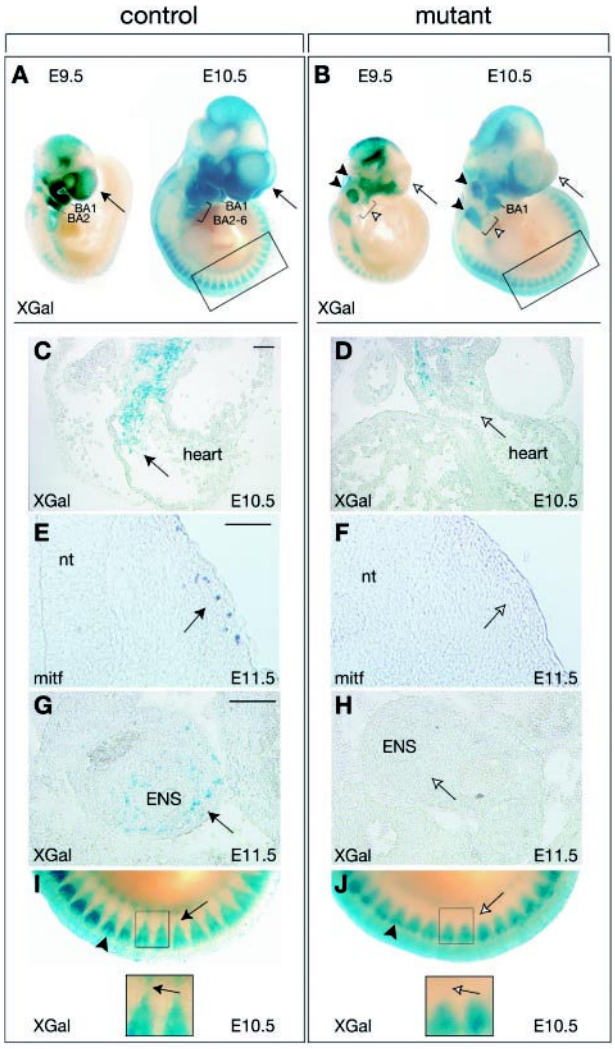

Fig. 1. In vivo fate mapping of NCSCs expressing stabilized β-catenin. (**A** and **B**) Neural crest cells and their progeny expressing β-galactosidase were revealed by whole mount XGal stainings (*13*). Mesenchymal neural crest in the nasofrontal region is present in control (arrows) and absent in mutant embryos (open arrows). Arrowheads point to prominent ganglion-like structures; open arrowheads indicate missing branchial arches (BA) in mutants. (**C** to **J**) XGal stainings and in situ hybridization on transverse sections show absence or reduction of cardiac neural crest, mitf-positive melanoblasts, and the enteric nervous system (ENS) in the mutant. Peripheral nerves [(I) and (J), enlarged areas marked by boxes in (A) and (B), respectively] were also missing. Arrows illustrate presence of and open arrows absence of neural crest derivatives. Arrowheads in (I) and (J) denote presence of DRG in control and mutant, respectively. nt, neural tube. Scale bars, 50 μm.

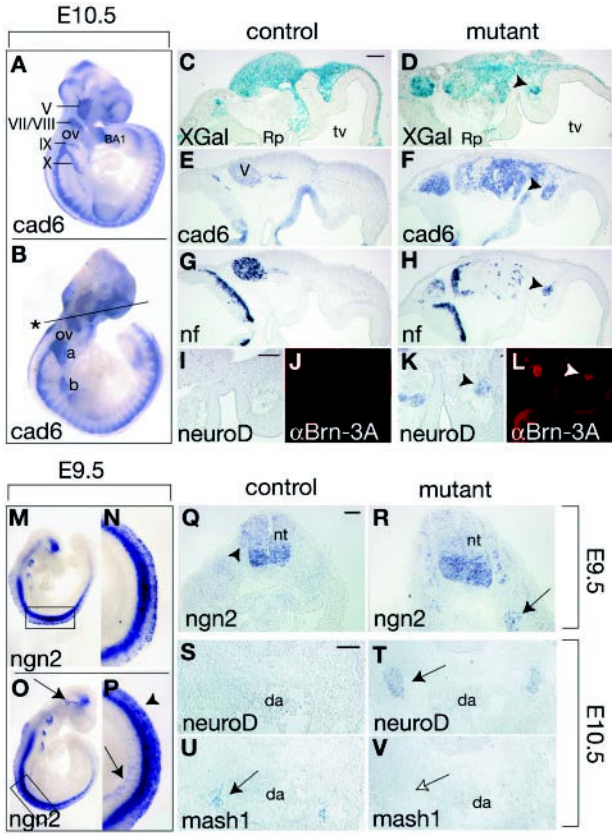

Fig. 2. Sensory neurogenesis at ectopic locations in mutant embryo. (**A** and **B**) cad6 expression at E10.5 reveals ganglia with sensory neuronal features in the mutant (B) formed instead of cranial nerves IX and X (a) and at the location of normal superior cervical ganglia (b) (see also fig. S2). V, VII/VIII, XI, and X designate cranial ganglia and nerves in the control (A). Ov, otic vesicle; BA, branchial arch. (**C** to **L**) Transverse sections cut at level (*) in (B). Note persistent expression of cad6 (F) in area of XGal-positive neural crest cells (D) and ectopic expression of nf in mutant (H). Ectopic neuronal cells (arrowheads) express the sensory markers neuroD (K) and Brn-3A (L). Rp, Rathke's pouch; tv, telencephalic vesicle. Scale bars, 100 μm. (**M** to **P**) In situ hybridization experiments show ectopic ngn2 expression in cranial [arrow in (O)] and ventral [arrow in (P)] regions in the mutant and increased expression in mutant migratory crest [arrowhead in (P)]. Control, (M) and (N); mutant, (O) and (P). (**Q** and **R**) On transverse sections at E9.5, ngn2-positive neural crest cells are restricted to the DRG anlage in control embryos [arrowhead in (Q)], whereas they spread ventrally in the mutant [arrow in (R)]. (**S** to **V**) At E10.5 in the mutant, neuroD-positive sensory neural cells [arrow in (T)] are found at the expense of mash1-positive autonomic neuronal cells [arrows in (U) and (V)]. da, dorsal aorta. Scale bars, 50 μm.

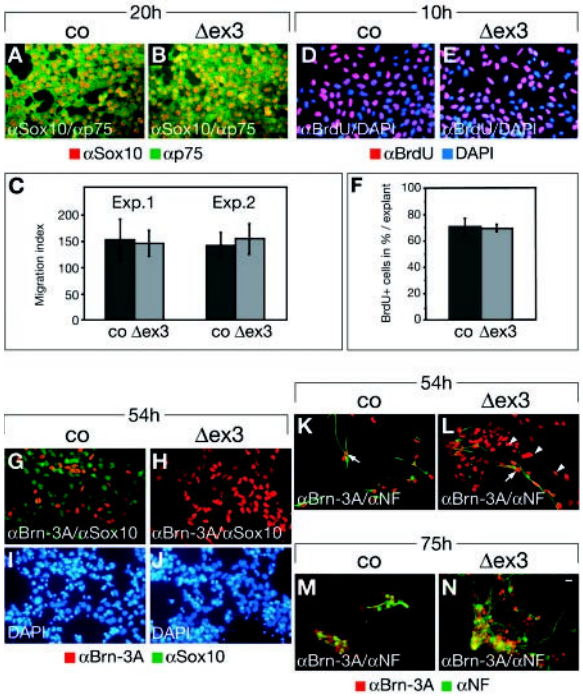

Fig. 3. Stabilized β-catenin does not affect emigration and proliferation of neural crest cells but promotes sensory neurogenesis. (**A** to **C**) Quantification of the migration index (*13*) did not reveal differences in migration of p75/Sox10-positive neural crest cells in control (co) and mutant (Δex3) explant cultures ($P > 0.2$). (C) Each bar represents the migration index (mean ± SD) of three different explants. (**D** to **F**) The number of proliferating, bromodeoxyuridine (BrdU)–incorporating cells was not significantly different between control and mutant cultures ($P > 0.75$). Three explants per genotype were analyzed, scoring 500 to 1000 cells per explant (F). Each bar represents the mean ± SD (**G** to **J**) Upon differentiation, mutant neural crest cells lose Sox10 expression and stain for the sensory marker Brn-3A, whereas many control NCSCs maintain Sox10 expression (table S1). (**K** to **N**) Differentiation is delayed in the mutant, because many Brn-3A–positive sensory neuronal cells (arrowheads) do not express NF (arrows) in the mutant at 54 hours (table S1). After prolonged incubation for 75 hours, however, most mutant cells are able to undergo full differentiation. Scale bar, 10 μm.

els: Wnt/β-catenin might promote the expansion of a sensory progenitor that has segregated from a neural crest cell with autonomic and other potentials (*20*, *25*, *26*). Alternatively, Wnt/β-catenin might have an instructive influence on fate decisions in an early NCSC able to generate sensory and autonomic neurons, glia, smooth muscle, and possibly other neural crest derivatives (*27*–*30*). The latter model is supported by the fact that in vivo sensory cells are not just expanded but rather generated at the expense of virtually all other neural crest lineages upon sustained β-catenin activation (Figs. 1 and 2). To rigorously distinguish between these models, we challenged early neural crest cells at clonal density with Wnt1 and, on sister plates, with different growth factors previously shown to promote specific fates in NCSCs (*31*) (Fig. 4, E to J; table S2). In control cultures without instructive growth factors, 88 ± 6.7% of all prospectively identified NCSCs generated mixed clones containing autonomic neurons and glia (*19*). In agreement with previous studies (*31*), BMP2 induced Mash-1–dependent autonomic neurogenesis in 90.5 ± 3.7% of all NCSCs, whereas upon transforming growth factor–β (TGF-β) treatment, about 70% of the cells adopted a smooth muscle–like fate. In the presence of Wnt1, 79.6 ± 2.5% of all NCSCs generated clones containing Brn-3A–positive sensory neurons. Most of these (95.4 ± 3.4%) were sensory neuron–only clones, which were not associated with Sox10 staining. The clone size of Wnt1-treated NCSCs was small, with many NCSCs giving rise to a single sensory neuron (Fig. 4, G and K). Furthermore, cell death was minimal in all clonal experiments (table S2), excluding selective effects of the factors added. The combined data indicate that Wnt signaling does not induce proliferation of a restricted sensory progenitor but rather promotes sensory fate decision in multipotent eNCSCs.

In vivo, members of the Wnt family specify neural crest from early dorsal neuroepithelial cells (*9*). Furthermore, the effects of ablation of *wnt1* and *wnt3* suggest a role of Wnt signaling in expansion of dorsal neural tube cells, including the premigratory neural crest (*10*). Our present data, together with the β-catenin loss-of-function analysis (*11*), indicate a subsequent function of Wnts in neural crest cells as they emigrate. Although the sensory lineage segregates early in PNS development (*20*, *25*, *26*), emigrating neural crest cells initially represent a population of stem cells (eNCSCs) that are homogeneous with respect to many developmental potentials, including sensory, autonomic, glial, smooth muscle–like, and possibly mesenchymal and other lineage formation (Fig. 2) (*27*). Similar to other growth factors that promote alternative fates (*31*), Wnt/β-catenin induces sensory neurogenesis by acting instructively on these eNCSCs. The molecular context that allows Wnt signaling to regulate cell cycle progression in certain stem

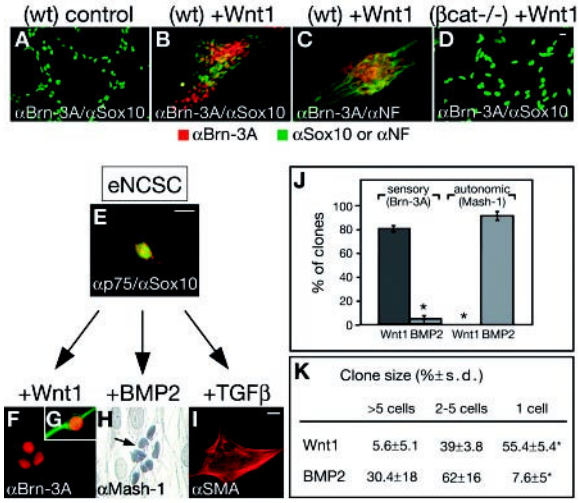

Fig. 4. Wnt1/β-catenin signaling instructs eNCSCs to adopt a sensory fate. (**A** to **D**) The effect of Wnt1 is β-catenin dependent. Wild-type (wt) NCSCs exposed to control monolayers fail to generate Brn-3A–positive sensory neurons (A), whereas wt NCSCs exposed to Wnt1 monolayers form ganglion-like cell aggregates containing Brn-3A/NF-positive sensory neurons [(B) and (C)]. β-catenin–deficient (β-cat$^{-/-}$) NCSCs are unable to generate sensory neurons, despite the presence of Wnt1 (D). Scale bar, 10 μm. (**E** to **J**) Clonal analysis demonstrates responsiveness of wt eNCSCs to different instructive growth factors including Wnt1. Of the p75-positive (red) founder cells, 90.3 ± 0.7% coexpressed Sox10 (green) (E). In the presence of Wnt1, founder cells generated clones of Brn-3A–positive sensory neurons (F) expressing NF (G). BMP2 instructed eNCSCs to generate Mash-1–expressing autonomic cells [arrow in (H)]. TGF-β induced a smooth muscle–like fate in most of the eNCSCs (I). Scale bars, 10 μm. (J) Quantification of clone composition (see also , fig. S2). (*) $P < 0.001$. The data are expressed as the mean ± SD of three independent experiments. Fifty to 150 clones were scored per experiment. (**K**) The cell number within individual Wnt1- and BMP2-treated clones was analyzed. Numbers (percentage of all clones per condition) are shown as the mean ± SD of three independent experiments, scoring 50 to 150 clones per experiment. (*) $P < 0.01$.

cells (*1*–*3*, *5*, *6*) and fate decision processes in NCSCs awaits investigation.

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32. Supported by grants of the Swiss National Science Foundation (to L.S. and U.S.) and by the National Center of Competence in Research "Neural Plasticity and Repair." We thank N. Mantei and R. Cassada for critical reading of the manuscript, A. McMahon and A. Berns for providing transgenic animals, and E. Ehler for advice with confocal microscopy. We acknowledge M. Wegner and J. Lee for riboprobes, and M. Wegner and E. Turner for antibodies.

Supporting Online Material
www.sciencemag.org/cgi/content/full/1091611/DC1
Materials and Methods
Figs. S1 and S2
Tables S1 and S2
References

17 September 2003; accepted 1 December 2003
Published online 8 January 2004;
10.1126/science.1091611
Include this information when citing this paper.

Anterior Cingulate Conflict Monitoring and Adjustments in Control

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Conflict monitoring by the anterior cingulate cortex (ACC) has been posited to signal a need for greater cognitive control, producing neural and behavioral adjustments. However, the very occurrence of behavioral adjustments after conflict has been questioned, along with suggestions that there is no direct evidence of ACC conflict-related activity predicting subsequent neural or behavioral adjustments in control. Using the Stroop color-naming task and controlling for repetition effects, we demonstrate that ACC conflict-related activity predicts both greater prefrontal cortex activity and adjustments in behavior, supporting a role of ACC conflict monitoring in the engagement of cognitive control.

A major goal of cognitive neuroscience is to understand the precise neural mechanisms that underlie cognitive control (*1*). An important question about the nature of cognitive control is how do the processes involved in implementing control become engaged, or in other words, what controls control (*2*)? One partial answer comes from the conflict hypothesis, which posits that monitoring of response conflict acts as a signal that engages control processes that are needed to overcome conflict and to perform effectively (*3*, *4*). Two brain regions that have been associated with cognitive control processes are the ACC and the prefrontal cortex (PFC). Although it is commonly accepted that the PFC is involved in implementing control (*5*–*7*), there have been differing hypotheses regarding the contribution made by the ACC (*8*–*10*). One of these, the conflict hypothesis, contends that a function of ACC is the monitoring of processing conflict (*3*, *11*). However, this has recently been challenged on two grounds: first, failure to find behavioral evidence for trial-to-trial adjustments in control following conflict when stimulus repetitions

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MATERIALS AND METHODS

Mating scheme and genotyping

wnt1-Cre mice were mated with *ß-catenin*$^{flox(ex3)/+}$ animals or, in order to perform in vivo fate mapping experiments, with *ß-catenin*$^{flox(ex3)/+}$*/ACZL* mice. Embryos that inherited *wnt1-Cre* and a *ß-catenin*$^{flox(ex3)}$ allele are referred to as mutant embryos, while littermates which inherited the incomplete combination of the above alleles served as control animals. The *wnt1-Cre* and the *ACZL* transgenes and the Δex3 allele were identified as described (*S1, S2*). Genotyping for the *ß-catenin*$^{flox(ex3)}$ allele was performed by PCR using primers ßcatEx2s (GACACCGCTGCGTGGACAATG) and ßcatEx3as (GTGGCTGACAGCAGCTTTTCTG) for 30 cycles of 94°C for 1 min, 65°C for 1min, and 72°C for 1 min. To obtain *ß-catenin*-deficient NCSCs, *wnt1-Cre/ß-catenin*$^{floxed/floxdel}$ mice were generated and genotyped as in (*S3*).

In vivo fate mapping experiments

The fate of control and mutant neural crest cells and their progeny was followed in compound transgenic *wnt1-Cre* animals bred to the ACZL mouse reporter line. In this reporter line, a floxed *lacZ* gene is restored and properly expressed upon Cre-mediated recombination (*S4*).

In situ staining procedures

Nonradioactive in situ hybridization with digoxigenin-labelled riboprobes, TUNEL labeling, and XGal staining for *lacZ*-reporter gene expression were performed on whole embryos or on cryosections as described (*S5, S6*). For immunohistochemistry, paraformaldehyde-fixed embryo sections were blocked in blocking solution (1% goat serum, 0.3% Triton-X-100 in PBS) for 1 h and incubated with polyclonal rabbit anti-Brn-3A antibody (diluted 1:1000) (*S7*) and a monoclonal mouse anti-Neurofilament160 (NF) antibody (diluted 1:500; Sigma) overnight at 4°C. Note that each marker was analyzed on at least 3 embryos per stage.

Cell culture

Mouse neural crest explant cultures under conditions permissive for sensory neurogenesis were prepared as reported (*S3*), with the exception that the cultures were incubated for 20 h at reduced oxygen levels (*S8*), which influences fate and survival of NCSCs. The neural tubes were used for PCR-genotyping. To allow full differentiation of mutant and control NCSCs, the defined medium (*S9*) was modified by adding the following factors after 50 h of culture: bFGF (4ng/ml); neurotrophin 3 (NT3, 6.25ng/ml), brain-derived nerve

growth factor (BDNF, 6.25 ng/ml), and retinoic acid (17.5ng/ml).

Wnt1-expressing and control NIH3T3 fibroblasts (*S10*) were irradiated (3000 rad) to abolish proliferation and plated as monolayers. To challenge neural crest explants with Wnt1, mouse neural tubes were isolated and plated onto the feeder cells to allow neural crest emigration directly onto the monolayers.

For clonal analysis, rat neural tubes were isolated at E10.5 and plated onto fibronectin-coated dishes as reported (*S11*). NCSCs were allowed to migrate for 15 h at low oxygen levels in defined medium according to (*S9*). NCSCs were replated (*S11*) either onto Wnt1-expressing monolayers, control monolayers, or pdL/fibronectin-coated dishes at clonal density. The plating efficiency was approx. 60 to 70%. 3 h after replating, living NCSCs were labelled for 30 min at RT using a rabbit anti-mouse p75 polyclonal antibody (1:300 dilution; Chemikon International), followed by a 30 min incubation with Cy3-conjugated goat anti-rabbit IgG (1:200 dilution; Jackson Immuno Research Laboratories), and single p75-positive clone founder cells were mapped (*S11*). Some culture dishes were fixed immediately after p75-staining and further processed for Sox10 immunostaining. Sister dishes with NCSCs on Wnt1-expressing layers were cultured in defined medium (*S9*) and analyzed after 3 d in culture. NCSCs on control layers and pdL/fibronectin-coated dishes were incubated in standard medium according to (*S12*). Some cultures on control monolayers were supplemented with 50ng/ml BMP-2 (R&D Systems) 2 d after plating and analyzed after an additional 24 h. Cultures on substrate-coated dishes were treated with 0.1ng/mlTGFß (R&D Systems) for 4 d.

Migration, proliferation, and survival assays of cultured cells

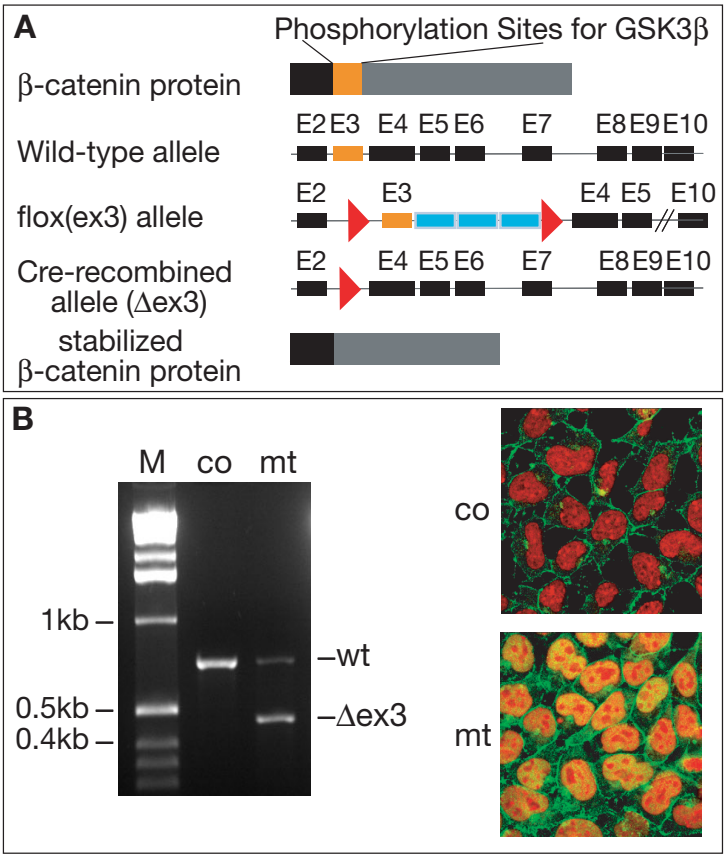
To quantify the neural crest outgrowth, digital images of explant cultures at 20 h were acquired and NIH image 1.62 software was used to measure the size of the explants. The outgrowth area (mm^2) was divided by the perimeter (mm) of the explant. This normalized number (in mm) is referred to as migration index and gives an estimate of the total outgrowth of a neural crest explant (*S3, S13*).

To quantify the percentage of proliferating cells during emigration, neural crest cells were treated with BrdU at 8 h as they migrated, and BrdU incorporation was analyzed at 10 h. To quantify the percentage of proliferating cells during differentiation, neural crest cells were treated with BrdU at 24 h, and BrdU incorporation was analyzed at 30 h. BrdU incorporation was assessed according to the manufacturer's instructions (Roche Diagnostics) and visualized using goat anti-mouse IgG conjugated to Cy3 (1:200 dilution; Jackson Immuno Research Laboratories).

Cell death was assayed by TUNEL as described (*S14*).

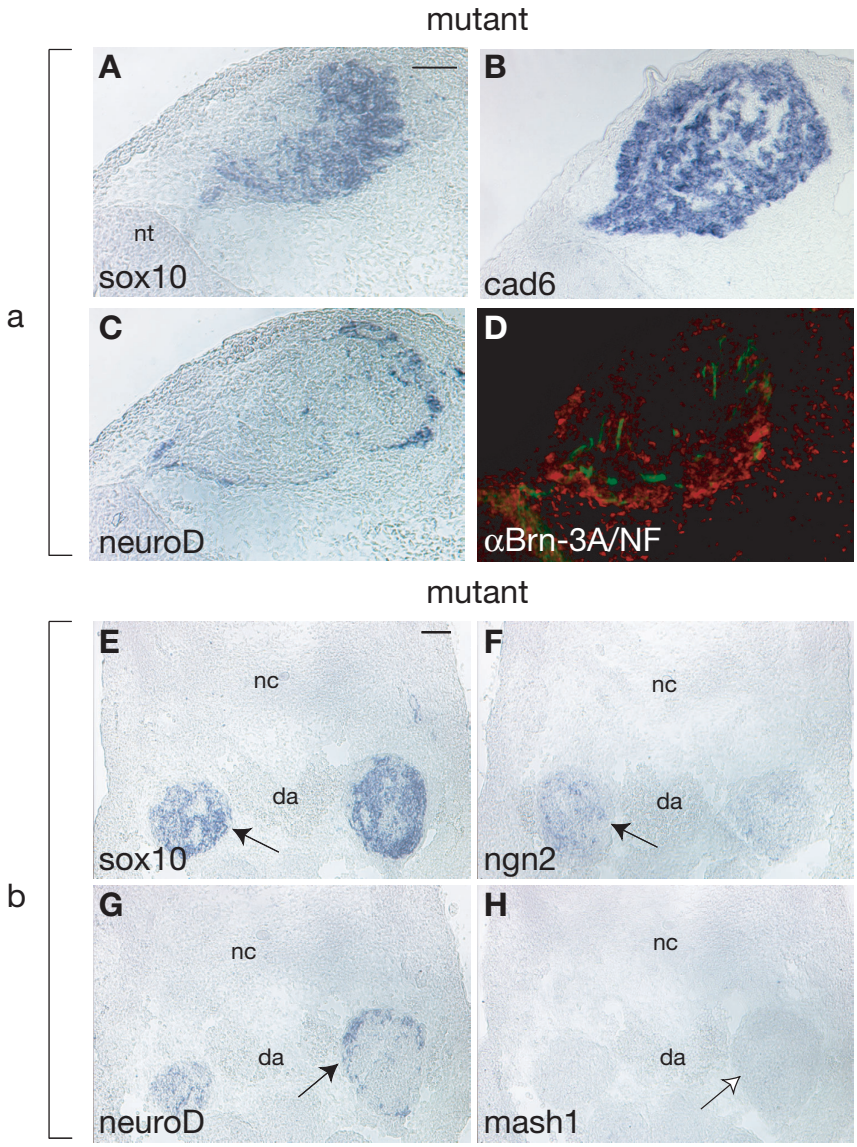
Immunocytochemistry

Anti-p75-, anti Sox10-, anti-Brn-3A-, and anti-NF160 antibody stainings were done as in (*S3*). Anti-ß-Catenin staining was performed using a polyclonal antibody (1:300 dilution; Sigma) and was combined with labelling by DRAQ5 (1:50 dilution; Biostatus Lt.). Smooth muscle actin was stained with a monoclonal anti-mouse SMA antibody (1:400 dilution, Sigma); for Mash-1 staining, cells were incubated with a monoclonal antibody (1:100 dilution, BD Biosciences) overnight at 4°C, followed by incubation with a HRP-coupled anti-mouse IgG antibody (1:200 dilution; DAKO) and by HRP development using DAB as substrate.



Supporting Fig. S1. Generation of mice expressing a stabilized form of ß-catenin in the neural crest. (**A**) Certain serine and threonine residues near the ß-catenin N-terminus are required for phosphorylation by GSK3ß and ensuing ubiquitin-mediated proteolysis (*S15*). The protein domain encompassing these phosphorylation sites is encoded by exon3 (orange box). Other exons are shown as filled boxes and the *neo* selection cassette (*S1*) in blue. Deletion or mutation of these phosphorylation sites results in stabilization of ß-catenin and, thus, its enhanced nuclear activity. Stabilized ß-catenin can be locally expressed by tissue-specific Cre-mediated recombination in animals carrying a *ß-catenin* allele in which exon3 is flanked by *loxP* sites (flox(ex3)) (triangles in red) (*S1*). To achieve this, *wnt1-Cre* mice (displaying Cre activity in virtually all neural crest cells (*S2, S16*)) were mated with *ß-catenin*$^{flox(ex3)/+}$ animals. (**B**) In those embryos that inherited both *Cre* and a *ß-catenin*$^{flox(ex3)}$ allele, exon3 of *ß-catenin* was successfully deleted in NCSCs, as shown by PCR analysis of genomic DNA isolated from cultured control (co) and mutant (mt) NCSCs. (wt), wild-type allele; (Δex3), deleted allele. M, DNA size marker. Anti-ß-Catenin immunostaining (green colour) combined with DRAQ5 nuclear labelling (red) show nuclear localization (yellow) of stabilized ß-Catenin in virtually all mutant NCSCs in culture. Single confocal planes are shown. Note that while none of the mutant embryos

survived to term, they were recovered alive at normal frequency (i.e. 50%) at embryonic day (E) 9.5 and at slightly lower frequency at E10.5 and E11.5. Thereafter, their frequency was markedly reduced. The sole mutant embryo found at E17 displayed severe craniofacial defects consistent with a developmental defect of cranial neural crest cells (*S17*).



Supporting Figure S2: Formation of ganglionic structures with sensory features in mutant embryos. (A-D) The prominent cranial ganglia that formed in the mutant instead of cranial nerves IX and X (see Fig. 1B; Fig. 2B (a)) contained many cells positive for sox10, cad6, neuroD mRNA, and Brn-3A (red) and NF (green) protein, as shown by staining on transverse sections at E10.5. (E-H) Similarly, ganglia with sensory features formed at the location of normal superior cervical ganglia (see (b) in Fig. 2B) and expressed the sensory lineage markers ngn1 (*S17*), ngn2, and neuroD. In contrast, the autonomic neuronal markers mash1 and ehand (*S17*) were not expressed. nc, notochord. Scale bars, 50 μm.

SUPPORTING TABLES

	% of all p75+ cells				% of all Brn-3A+ cells
	(20h)		(54h)		(54h)
	Sox10	Brn-3A	Sox10	Brn-3A	NF
co	100	0	70.3±6.3	29.7±6.3	100
Δex3	98.7±1.2	1.3±1.1	0	82.2±7.8*	18.7±9.9

	% of all cells		
	(10h)	(30h)	(30h)
	BrdU		TUNEL
co	70.9±6.4	36.6±3.1	1.23±0.4
Δex3	69.5±2.8	32.9±3.5	1.31±0.7

Supporting Table S1. Marker expression, proliferation, and cell death of neural crest cells expressing activated ß-catenin. Neural crest cells from either control or mutant (Δex3) mouse embryos were allowed to emigrate and differentiate in explant cultures (see also Fig. 3). Marker expression was assayed at 20 h and at 54 h. Note that 20 h after emigration, virtually all cells displayed features of NCSCs being positive for p75 and Sox10 but negative for the sensory differentiation marker Brn-3A. In conditions permissive for sensory neurogenesis, most cells in the control maintain Sox10 expression, while in the mutant, all cells lose NCSCs features and the majority adopts a sensory fate marked by Brn-3A (54 h). Proliferation and cell death during migration and differentiation (10 h; 30 h) are comparable in control and mutant explants. Thus, the mutant phenotype appears to be due to an altered developmental potential of neural crest cells rather than to selective mechanisms or a defect before emigration. In agreement with this, we did not detect neural tube deficiencies at the time of neural crest delamination (*S17*).

In control cultures at 54 h, 100% of all Brn-3A-positive cells displayed features of fully differentiated neurons, while many mutant Brn-3A cells were not expressing NF (see also Fig. 3K-N). Likewise, in vivo many Brn-3A-positive sensory neuronal cells were NF-negativ, and expression levels of trk neurotrophin receptors, differentiation markers for sensory neurons, were reduced in neural crest derivatives of mutant embryos (*S17*). Prolonged incubation in the presence of neurotrophins allowed mutant sensory cells to differentiate in culture, indicating that sustained ß-catenin activity delays rather than impedes neuronal differentiation.

All figures represent the mean±s.d. of three experiments. At least 200 cells were counted per experiment.

condition	†	sN	sN+G	G	aN
control	2±2	0	0	98±2	80.4±5.6
Wnt1	7.6±3.8	75.8±1	3.8±2.8	12.8±1.5	0
BMP2	9.5±3.7	4.5±3.9	0	15.3±2.9	90.5±3.7
TGFβ	34±21.4	0	0	0	0

Supporting Table S2. Clonal analysis of p75-positive eNCSCs in the presence of different instructive growth factors. Wild-type eNCSCs at clonal density were treated as described in Materials and Methods, and the progeny of single p75-positive cells was characterized by immunocytochemistry for Brn-3A and Sox10 or, independently, for Mash-1. The numbers indicate the phenotype of the progeny in %±s.d. '†' designates lost clones. A colony was labeled 'sN' (sensory neuron), 'G' (presumptive glia), or 'aN' (autonomic neuron) when at least one cell was expressing the corresponding marker. Note that in control conditions, 85.5±2.0% of the 'G'-clones are associated with NF (*S17*). This, together with the Mash-1 staining, indicates that most clones in the control are mixed 'aN+G'. In the presence of TGFß, all surviving clones were composed of smooth muscle-like cells (*S17*). The low numbers of lost clones demonstrate that Wnt1, BMP2, and TGFß are not selectively eliminating certain cell lineages but are acting instructively on eNCSCs. The numbers represent the mean±s.d. of three experiments, scoring 50 to 100 clones per experiment.

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4.2 Temporal control of neural crest lineage generation by Wnt/ β-catenin signaling

Submitted to Development, 2.8.2011
(Current status: in revision)

My contribution to this work:

- Designing and performing all the experiments
- Collection of all the Data, with some help of our laboratory technician I. Miescher under my supervision
- Generation of all the Figs.
- Generation of most of the reagents, including breeding, genotyping of the mice and collection of the embryos, with some help of I. Miescher under my supervision
- Design of the paper outline, together with L. Sommer. Writing of “Figure Legends” and “Materials and Methods”. Discussion and modification of the manuscript.

Temporal Control of Neural Crest Lineage Generation by Wnt/β-Catenin Signaling

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RUNNING TITLE

βCatenin in melanocyte formation

KEY WORDS

Wnt, β-Catenin, neural crest stem cells, sensory neurons, melanocytes

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SUMMARY

Wnt/β-Catenin signaling controls multiple steps of neural crest development, ranging from neural crest induction, lineage decisions, and differentiation. In mice, conditional *β-Catenin* inactivation in premigratory neural crest cells abolishes both sensory neuron and melanocyte formation. Intriguingly, the generation of melanocytes is also prevented by activation of *β-Catenin* in the premigratory neural crest, which promotes sensory neurogenesis at the expense of other neural crest derivatives. This raises the question of how Wnt/β-Catenin signaling regulates the formation of distinct lineages from the neural crest. Using various *Cre* lines to conditionally activate *β-Catenin* in neural crest cells at different developmental stages, we show that neural crest cell fate decisions *in vivo* are subject to temporal control by Wnt/β-Catenin. Unlike in premigratory neural crest, *β-Catenin* activation in migratory neural crest cells promotes the formation of ectopic melanoblasts, while production of most other lineages is suppressed. Ectopic melanoblasts emerge at sites of neural crest target structures and in many tissues usually devoid of neural crest-derived cells. *β-Catenin* activation at later stages, in glia, or in specified melanoblasts does not lead to surplus melanoblasts, indicating a narrow time window of Wnt/β-Catenin responsiveness during neural crest cell migration. Thus, neural crest cells appear to be multipotent *in vivo* both before and after emigration from the neural tube but adapt their response to extracellular signals in a temporally controlled manner.

INTRODUCTION

Many neural and non-neural cell types in vertebrates are produced by the neural crest, a transient embryonic structure that emerges during neurulation at the dorsal part of the neural tube (Le Douarin et al., 2008). Among the neural crest derivatives are the pigment cells in the skin reported to originate either directly from neural crest cells migrating from the neural tube on a dorsolateral pathway or from nerves innervating the skin (Sommer, 2011). Melanocyte lineage specification of dorsolaterally migrating cells is thought to occur already at an early stage of neural crest development. Cells fated for the melanocyte lineage stall in the so-called migration staging area (MSA) adjacent to the neural tube before continuing their dorsolateral migration (Weston, 1991). In the MSA, melanocyte specification is indicated by expression of the Microphthalmia-associated transcription factor Mitf that controls melanoblast development and survival (Opdecamp et al., 1997). Even though Mitf is crucial in melanocyte development, it is not the only factor required for melanocyte specification as some melanoblast are initially formed in homozygous *Mitf*-mutant mice (Hornyak et al., 2001). Both in cell culture and *in vivo,* expression of *Mitf* is regulated by canonical Wnt signaling, indicating a central role of this signal transduction pathway in melanocyte development (Dorsky et al., 2000; Takeda et al., 2000; Hari et al., 2002; Widlund et al., 2002). Canonical Wnt signaling involves the intracellular signaling component β-Catenin that upon its activation translocates to the nucleus and induces specific transcriptional responses (Gordon and Nusse, 2006). In cultures of mouse and quail neural crest cells, treatment with Wnt or activation of β-Catenin enhanced melanoblast proliferation and differentiation (Dunn et al., 2000; Jin et al., 2001). More strikingly, constitutive activation of β-Catenin in zebrafish *in vivo* promoted the formation of melanocytes while repressing neural cell

lineages (Dorsky et al., 1998). In contrast, upon inhibition of Wnt signaling, neural crest cells adopted a neural rather than a pigment cell fate. Similarly, in the mouse, conditional deletion of *β-Catenin (Ctnnb1)* in premigratory neural crest cells also prevented the generation of melanocytes (Hari et al., 2002). Unlike in zebrafish, inactivation of *β-Catenin* in the mouse neural crest was not only required for melanocyte formation but also for sensory neurogenesis (Hari et al., 2002). However, in contrast to zebrafish, β-Catenin signal activation in the mouse neural crest *in vivo* did not enhance melanocyte formation, but rather promoted wide spread formation of sensory neurons at the expense of virtually all other possible neural crest cell fates, including melanocytes (Lee et al., 2004). In the present study we offer an explanation for these inconsistent findings, showing that in mouse embryos, Wnt/β-Catenin signaling controls sensory fate acquisition before regulating melanocyte lineage formation. To analyze the temporal control of neural crest lineage generation by Wnt/β-Catenin signaling, we genetically manipulated *β-Catenin* at different stages of neural crest and early melanocyte development. Our results indicate that sensory neuronal and melanocyte lineages are successively generated from neural crest cells by sequential β-Catenin signaling.

MATERIALS AND METHODS

Animals and genotyping. The *Cre/loxP* system was used to conditionally express a stabilized form of β-Catenin as described in Lee et al. 2004. Mutant embryos inherited one *Cre* allele and one allele of *β-Catenin (Ctnnb1)$^{\Delta ex3}$*, while littermates inheriting incomplete combination of alleles served as control animals. For neural crest-specific mutations of *Ctnnb1$^{\Delta ex3}$* either *Wnt1-Cre* (Danielian et al., 1998) or *Sox10-Cre* (Matsuoka et al., 2005) transgenic mouse lines were used. Expression of stabilized β-Catenin in the glial lineage was achieved by using *Dhh-Cre* mice (Jaegle et al., 2003) or *Plp-CreERT2* mice (Leone et al., 2003), for expression in melanoblasts the *Tyr-CreERT2* (Bosenberg et al., 2006) mouse line was used. Genotyping for all *Cre* alleles was performed by PCR using primers Cre-lo (AGGCTAAGTGCCTTCTCTACAC) and Cre-up (ACCAGGTTCGTTCACTCATGG); 35 cycles of 94℃ for 45s, 58℃ for 30s, 72℃ for 1 min. *In vivo* fate mapping experiments were done by crossings with either ROSA26 reporter (R26R) (Soriano, 1999) or Z/EG (Novak et al., 2000) mouse lines.

Inducible Cre Expression. For activation of the CreERT2 protein Tamoxifen (TM; Sigma T5648) was dissolved in an ethanol/ sunflower oil mixture (1:10) at 10 mg/ml. 1 mg Tamoxifen was injected intraperitoneally into the pregnant mother on one day for *Plp-CreERT2* mouse lines and on two consecutive days for *Tyr-CreERT2* mouse lines.

***In situ* hybridization, X-Gal staining and Immunohistochemistry.** In situ hybridization on cryo sections with digoxigenin-labeled riboprobes were performed as described (Hari et al., 2002). For detection of *LacZ*-reporter gene expression using X-Gal staining, cryosections were fixed for 5 min on ice in 2% FA, 0.2% Glutaraldehyde, 0.02% NP40, in PBS, washed twice in PBS and stained for 4-16 h at 37℃ in staining solution (1 mg/ml X-Gal, 10mM K3Fe (CN)6; 10mM K4Fe(CN)6; 2mM MgCl2; 0.02% NP40, in PBS). Immunohistochemistry was performed on cryo sections. Sections were fixed for 5 min in 4% Formaldehyde at RT and treated with blocking buffer (10% goat serum, 0.3% Triton, 0.1% BSA in PBS) for 30 min, except for anti-Sox10 staining, where blocking buffer with 1% BSA instead of 10% goat serum was used. Primary antibodies were diluted in blocking buffer and used at the following concentrations: mouse anti-TH (1:200, Sigma T1299), goat anti-Sox10 (1:200, Santa Cruz sc-17342), rabbit anti-GFP (1:500, Abcam ab290), rabbit anti-Brn3a (1:2000, gift from E.Turner, University of Washington). All primary antibodies were detected by immunofluorescence with secondary antibodies from Jackson Immunoresearch or Invitrogen.

Statistical Analysis. Quantifications were done on at least three pairs of control vs. mutant embryos. For each embryo, cells in a specific area were counted on three or more different sections. All quantifications were done on transverse sections. For Fig. 2R, *Dct*-positive cells were counted in at least 19 MSAs on at least 10 sections per embryo at E11.5. On average 30 sections of MSAs per embryo were used for quantification. To quantify melanoblasts in the skin at E16.5, all *Dct*-positive cells on one lateral side (dorsal to ventral) of the skin were counted on 6 sections. The

number of *Dct*-positive cells was normalized to the length of the skin area counted. For Fig. 4E, Brn3a- and *Mitf*-positive cells were counted on at least 7 sections of the sympathetic ganglia (on average, cells on 12 sections were counted). Quantifications were done on 4 *Wnt1-Cre/Ctnnb1*$^{\Delta ex3}$ embryos and on 4 *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ embryos. Quantification of *Mitf*- and Brn3a-positive cells was done on adjacent or near- adjacent sections of the same embryos. Results are shown as mean ± standard deviation of the mean (SD). Statistical analysis (two-tailed unpaired Student's t test; calculation of SD) were done with Microsoft Excel.

RESULTS

β-Catenin signal activation in premigratoy, but not in migratory neural crest cells suppresses melanocyte specification

Although in mice, neural crest cells concomitantly engage in the ventral and dorsolateral migratory pathway (Serbedzija et al., 1990), neural and melanocyte cell type specification might occur sequentially. This is reflected by the sequential expression of transcription factors associated with lineage specification: *Neurogenin 2 (Neurog2)*, a basic helix-loop-helix (bHLH) transcription factor required for sensory neuogenesis (Ma et al., 1996), is already expressed in neural crest cells as they begin to delaminate from the neural tube (Sommer et al., 1996). In contrast, the bHLH leucine zipper transcription factor *Mitf* that promotes melanoblast development is only expressed from E10.5 onwards (Opdecamp et al., 1997). Accordingly, *Neurog2* was prominently expressed in migrating neural crest cells and forming dorsal root ganglia (DRG) in caudal regions of mouse embryos at embryonic day

(E)10.5, while *Mitf* was not yet expressed at this stage (Fig. 1A, B). At E11.5, however, *Mitf* expression was readily detectable in cells close to the caudal neural tube (Fig. 1C).

To address whether the sequential appearance of sensory and melanocyte lineage markers might involve reiterative β-Catenin signaling, we aimed to genetically manipulate β-Catenin activation in a temporally controlled manner using the *Cre-loxP* system. In *Wnt1-Cre* transgenic mice, *Cre* is expressed in the dorsal neural tube at stages before neural crest delamination (Danielian et al., 1998; Jiang et al., 2000; Hari et al., 2002). Therefore, Cre recombinase in these mice is active in the entire premigratory neural crest population and its derivatives, as demonstrated by *Cre*-induced β-galactosidase expression using the *ROSA26 Cre* reporter line (Soriano, 1999) (Fig. 1D, F, H). Similarly, all progeny of Cre-expressing neural crest cells are labeled in *Sox10-Cre* animals (Matsuoka et al., 2005) (Fig. 1E, G, I). Unlike *Wnt1-Cre*, however, *Sox10-Cre* marks neural crest cells only after their emigration from the neural tube, as shown by staining for Cre reporter-driven β-galactosidase expression both on whole mount embryos and on transversal sections at E9.5 and E10.5 (Fig. 1E-I).

The sequential Cre expression in *Wnt1-Cre* and *Sox10-Cre* animals allowed us to address a potential stage-dependent role of canonical Wnt signaling during neural crest development. Previously, we have demonstrated that expression of a constitutively active stabilized form of β-Catenin in the premigratory neural crest of

Wnt1-Cre/Ctnnb1$^{\Delta ex3}$ mice promotes sensory neurogenesis at the expense of virtually all other neural crest derivatives, including melanocytes (Lee et al., 2004). Accordingly, cells expressing *Mitf* (Lee et al., 2004) or the early melanocyte marker Dopachrome tautomerase *(Dct)* (Fig. 2A-D) were absent in the migratory staging area (MSA) and around the otic vesicle (OV) of *Wnt1-Cre/Ctnnb1*$^{\Delta ex3}$ embryos at E11.5. In contrast, melanoblasts were present in *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ embryos at E11.5, as revealed by *in situ* hybridization analysis of *Mitf* and *Dct* mRNA (Fig. 2E-M). Likewise, melanocytes expressing stabilized β-Catenin were able to colonize the skin and hair follicle primordia at E16.5, similar to control melanocytes (Fig. 2N, P). *In vivo* fate mapping using the *ROSA26* reporter line confirmed that melanocytic cells in the skin of *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ embryos were recombined to an extent comparable to that in the control (Fig. 2O, Q, arrows). Quantification of *Dct*-positive cells at E11.5 in the MSA and at E16.5 in the skin further demonstrated the presence of normal numbers of melanoblasts despite constitutive expression of β-Catenin (Fig. 2R). Thus, while β-Catenin signal activation in premigratory neural crest prevents pigment cell formation, it does not interfere with melanocyte lineage specification and localization of melanocytes to the OV and the skin, if activated only once neural crest cells have emigrated from the neural tube.

β-Catenin signal activation in migratory neural crest cells prevents formation of multiple neural and non-neural derivatives of the neural crest

The normal generation of orthotopic melanocytes in *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ embryos raised the question of whether constitutive β-Catenin activity affects neural crest cell

fates only at developmental stages of premigratory, but not migratory neural crest cells. To address this issue, we further analyzed the phenotype of *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ embryos, focusing on various neural crest derivatives that fail to properly form in *Wnt1-Cre/Ctnnb1*$^{\Delta ex3}$ animals (Lee et al., 2004). In the head, *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ displayed major gross morphological anomalies from E13.5 onwards, with varying degrees of malformations and reduced population of craniofacial structures by neural crest-derived cells, as shown by *in vivo fate* mapping at E14.5 (Fig. 3A-C). Furthermore, eGFP expression upon *Sox10-Cre*-mediated recombination in Z/EG reporter mice (Novak et al., 2000) revealed strongly reduced formation of peripheral nerves due to β-Catenin signal activation (Fig. 3D, E). Similarly, the enteric nervous system (ENS) was completely lacking in *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ embryos, with virtually no eGFP-positive neural crest-derived cells populating the mutant gut at E11.5 and E16.5 (Fig. 3F-I). Finally, the number of autonomic neurons expressing tyrosine hydroxylase (TH) was drastically reduced in *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ animals at the sites where sympathetic ganglia are normally found (Fig. 3K-N). These data demonstrate that β-Catenin signaling interferes with the generation of several neural crest-derived cells types, even if activated only after neural crest cell emigration.

β-Catenin signal activation in migratory neural crest cells leads to formation of ectopic melanoblasts and melanocytes

In *Wnt1-Cre/Ctnnb1*$^{\Delta ex3}$ mice, activation of β-Catenin signaling in premigratory neural crest cells results in the generation of ectopic, often large-sized sensory ganglia (Lee

et al., 2004). At E10.5, such oversized ganglia can be found, for instance, at the location of normal sympathetic ganglia, containing undifferentiated Sox10-positive cells, *Neurog2*-positive sensory progenitors, and Brn3a-expressing differentiated sensory neurons (Fig. 4G, H) (Lee et al., 2004). In contrast, although ectopic Brn3a-positive cells were still present at the sites of sympathetic ganglia, the size of ganglia was smaller in *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ than in *Wnt1-Cre/Ctnnb1*$^{\Delta ex3}$ embryos (*$p < 0.05$) (Fig. 4G-K). Intriguingly, however, while in *Wnt1-Cre/Ctnnb1*$^{\Delta ex3}$ mice only few *Mitf*-positive melanoblasts were present at sites where sympathetic ganglia usually reside, *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ embryos exhibited significantly more melanoblasts in these ganglionic structures at E10.5 (Fig. 4B-E, L). The relative number of Brn3a-expressing cells was reduced accordingly in ganglia of *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ embryos (Fig. 4L). In addition to *Mitf*, some of the ectopic cells already expressed *Dct*, confirming their melanocytic identity (data not shown). Neither Brn3a- nor *Mitf*-positive cells were expressed in the control ganglia, which were much smaller in size as compared to the mutants (Fig. 4A, F). The concurrent suppression of autonomic neurogenesis and other neural crest cell lineages in these embryos (Fig. 3) suggests that β-Catenin signaling can influence neural crest cell fate decisions after their emigration from the neural tube.

To investigate whether ectopic melanocytes in *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ embryos are restricted to sympathetic ganglia or more broadly found, we systematically analyzed various mutant embryo structures for the presence of cells expressing melanoblast markers. *In situ* hybridization analysis of *Mitf* mRNA expression at E10.5 revealed many melanocytic cells not just within sympathetic ganglionic anlagen, but also

dispersed around these structures (Fig. 5A, B). Strikingly, at subsequent developmental stages melanocytic cells were detected at several ectopic places in *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ embryos, including in many tissues that usually do not harbor neural crest derivatives. For instance, at E12.5, the kidney primordium comprised *Dct*-expressing cells, which were shown by *in vivo* fate mapping to be of neural crest origin (Fig. 5C-F). Moreover, many neural crest-derived *Dct*-positive cells were spread throughout the diaphragm at E14.5, giving the appearance of cells migrating along the border to the liver and other inner organs (Fig. 5G-K). Likewise, in the spleen and the urogenital tract at E16.5, many melanocytic cells were detectable, either dispersed throughout the organs or sometimes even integrated in epithelial structures (Fig. 5L-S). Of note, several of the cells marked by *Dct* expression were also displaying pigmentation, demonstrating their melanocyte identity (Fig. 5N, R, insets). Ectopic melanoblasts were also found at further locations, such as for example in reproductive organs (testes and ovaries) and around the dorsal aorta (data not shown). As confirmed by Cre-mediated genetic cell tracking, all these organs were devoid of neural crest-derived cells in the control. Thus, expression of a constitutively active form of β-Catenin in migratory neural crest cells leads to ectopic localization of melanoblasts and differentiated pigment cells in various embryonic organs.

Ectopic melanocyte formation is due to β-Catenin signal activation in migratory neural crest cells rather than in committed melanoblasts or glial cells

The emergence of extra melanocytes, concomitant with the loss of other neural crest derivatives in *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ embryos, is consistent with the idea that β-

Catenin signal activation regulates cell fate choices in multipotent migratory neural crest cells, promoting melanocyte formation at the expense of other fates. Alternatively, β-Catenin signal activation might cause an expansion of melanoblasts on their way from the MSA to the skin, leading to the ectopic localization of excessively produced cells. To exclude this alternative explanation for the presence of ectopic melanoblasts, we made use of the inducible *Tyr-CreERT2* allele that drives Cre recombinase in neural crest cells only after dorsolateral migration and melanocyte fate specification (Bosenberg et al., 2006). Induction of *Ctnnb1*$^{\Delta ex3}$ expression by treatment of the pregnant females with tamoxifen at E11.5 and E12.5 (see Material and Methods) resulted in recombination in melanocytes in the skin and in forming hair follicle primordia at E16.5 (Fig. 6B). Consistent with a previous report (Delmas et al., 2007), β-Catenin signal activation appeared not to result in an overt increase in melanoblast cell numbers in the skin (data not shown), speaking against a role of β-Catenin signaling in promoting melanoblast proliferation. Of note, we did not observe any ectopic melanoblasts in *Tyr-CreERT2/Ctnnb1*$^{\Delta ex3}$ animals, as demonstrated by *in vivo* fate mapping and *in situ* hybridization analysis of *Dct* expression (Fig. 6C,D, illustrating absence of melanoblasts in the spleen, as an example). These data rule out the possibility that ectopic melanoblasts in *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ animals are produced as a consequence of increased orthotopic melanoblast proliferation.

Recently, it has been reported that melanocytes not only arise from neural crest cells migrating along the dorsolateral pathway, but also from Schwann cell precursors (SCPs) located in nerves innervating the skin (Adameyko et al., 2009). To address whether melanocyte fate acquisition at ectopic places could result from elevated β-

Catenin signaling in nerves, we expressed *Ctnnb1*$^{\Delta ex3}$ specifically in peripheral glia by means of *Dhh-Cre*-mediated recombination (Jaegle et al., 2003). Although recombination in nerves appeared to be efficient in *Dhh-Cre/Ctnnb1*$^{\Delta ex3}$ embryos from E12 onwards (Jaegle et al., 2003), and data not shown), we neither observed recombined orthotopic melanocytes (Fig. 6F, open arrow) nor melanoblasts at ectopic locations in these animals at E16.5 (Fig. 6G-H, data not shown). These findings do not support a role of β-Catenin in promoting the generation of melanocytes from nerve cells. Based on fate mapping experiments with inducible *Plp-CreERT2*, it has been suggested that the SCP-to-melanocyte transition occurs within a narrow time window during murine development around E11 (Ernfors, 2010). To exclude that *Dhh-Cre* activity might be too late to track and influence a SCP-to-melanocyte switch, we induced expression of *Ctnnb1*$^{\Delta ex3}$ at various early and late time points using the *Plp-CreERT2* line (Leone et al., 2003). Tamoxifen treatment of *Plp-CreERT2/Ctnnb1*$^{\Delta ex3}$ animals at E9.5 marked multiple neural crest derivatives by Cre–mediated recombination, including neuronal cells in the DRG (Fig. 6I) and in the enteric nervous system (data not shown) This indicates that at this early developmental stage *Plp-CreERT2* expression occurs in unspecified neural crest cells and is not restricted to SCPs. In agreement with the results obtained with *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ animals β-Catenin signal activation in *Plp-CreERT2/Ctnnb1*$^{\Delta ex3}$ embryos at E9.5 led to the appearance of ectopic melanoblasts at E16.5 (Fig. 6L, M). Cre activity in *Plp-CreERT2/Ctnnb1*$^{\Delta ex3}$ embryos became more restricted, however, upon induction at E11.5 and later stages (Fig. 6N, R). In contrast to *Dhh-Cre*, *Plp-CreERT2* efficiently marked nerves as well as melanoblasts in the skin when induced at E11.5, E12.5, E14.5 or at postnatal stages (Fig. 6K,O,S; data not shown) Importantly, when activated from E11.5 onwards, β-Catenin signaling failed to

promote ectopic melanoblast formation in *Plp-CreERT2/Ctnnb1*$^{\Delta ex3}$ animals (Fig. 6P,Q,T,U). These data indicate that β-Catenin-mediated generation of melanocytes at ectopic places is due to its activation in neural crest cells before they have localized to nerves or to the skin.

DISCUSSION

Canonical Wnt/β-Catenin signaling has been shown to play reiterative roles during neural crest development, sequentially controlling neural crest induction, neural crest cell delamination, lineage specification, and differentiation of neural crest derivatives (Garcia-Castro et al., 2002; Burstyn-Cohen et al., 2004; Lewis et al., 2004) (Kleber and Sommer, 2004; Steventon et al., 2009; Li et al., 2011). In the present study, we add a new twist to this theme, demonstrating that also the generation of distinct neural crest cell fates in the mouse is subject to sequential Wnt/β-Catenin signaling. Activation of β-Catenin in the premigratory neural crest promotes sensory neurogenesis while suppressing virtually all other possible lineages, including melanocytes (Lee et al., 2004). If β-Catenin signal activation occurs in migratory rather than premigratory neural crest cells, however, melanocytes represent a major cell type generated, while most other neural crest cell lineages still fail to develop. Thus, we propose that neural crest cell fate decisions are regulated by stage-dependent differential responsiveness to Wnt/β-Catenin signal activation.

Wnt/β-Catenin signaling regulating neural crest cell sensory and melanocyte lineage decisions

In previous reports, Wnt/β-Catenin signal activation in neural crest cells has either been associated with expansion of pigment cell numbers or with increased sensory neurogenesis (Dorsky et al., 1998; Jin et al., 2001; Lee et al., 2004). Differences in the outcome of these studies have been attributed, among others, to possible species-specific mechanisms controlling neural crest lineage decisions (Sommer,

2011). Our work provides an additional explanation for how to reconcile the divergent reports: The exact timing of Wnt/β-Catenin signal manipulation apparently has an influence on whether neural crest cells choose to produce sensory neurons or melanoblasts. The use of mouse lines expressing Cre recombinase at different stages of neural crest development allowed us to temporally control conditional *β-Catenin* activation: As demonstrated by *in vivo* fate mapping, the use of *Wnt1-Cre* results in recombination in the dorsal neural tube at stages when this structure comprises the premigratory neural crest cell population. In contrast, *Sox10-Cre* is not active in the dorsal neural tube and, therefore, in premigratory neural crest cells. Hence, genetic tracking of *Sox10-Cre*-mediated recombination fails to mark cells of the dorsal neural tube at any developmental stage. *Sox10-Cre* efficiently labels, however, neural crest cells after their emigration from the neural tube. The differential outcome of *Wnt1-Cre*- vs. *Sox10-Cre*-driven *β-Catenin* activation is, therefore, consistent with the idea that canonical Wnt signaling regulates sensory neurogenesis in neural crest cells before or during their emigration, when the cells are still localized in or close to the dorsal neural tube. In contrast, the melanocyte lineage is promoted after neural crest cell emigration. This view is also compatible with our previous finding that conditional inactivation of *β-Catenin* in the premigratory neural crest reveals a requirement of the Wnt pathway for both sensory neuron as well as pigment cell formation (Hari et al., 2002).

Although we show that β-Catenin signal activation is sufficient to influence neural crest cell lineage decisions in a stage-dependent manner, we were unable for technical reasons to demonstrate a stage-dependent requirement for *β-Catenin.*

Using Cre-lines active in specified melanoblasts and/or in Schwann cell precursors, we have defined a relatively narrow time window during which neural crest cells respond to *β-Catenin* activation by increased melanocyte formation (see below for further discussion): After neural crest cell emigration, but before the cells have reached the dorsolateral migratory pathway or the nerves. Since loss of protein lags behind conditional gene deletion, *Sox10-Cre*-dependent ablation of *β-Catenin* did not abolish canonical Wnt signaling during this narrow time window of signal responsiveness, and neural crest cells were found, therefore, to adopt a melanocytic fate in *Sox10-Cre/Ctnnb1*$^{flox/flox}$ animals (Hari L., and Sommer L., unpublished). However, the combined *in vivo* loss- and gain-of-function data, together with the observation that the Wnt/β-Catenin pathway is active in a fraction of migratory neural crest cells *in vivo* (data not shown), support a role of canonical Wnt signaling in controlling the melanocyte fate in migratory neural crest cells after their delamination from the neural tube.

Our findings raise the question of why premigratory and migratory neural crest cells are differentially responsive to Wnt/β-Catenin signal activation. Possibly, this might be due to cell-intrinsic differences displayed by neural crest cells depending on their location and the developmental stage (White et al., 2001; Bixby et al., 2002; Fuchs et al., 2009). The molecular nature of these intrinsic cues is largely unknown, but changes in growth factor receptor expression have been shown to modulate the response of neural crest cells to microenvironmental factors. With respect to Wnt signaling, cell type- and stage-specific responses have also been described for other systems than the neural crest (Kleber and Sommer, 2004). For instance, in the

central nervous system, Wnt regulates cell cycle progression at early stages of development, but induces neuronal differentiation at later time points (Chenn and Walsh, 2002; Hirabayashi et al., 2004). Apart from cell-intrinsic properties modulating Wnt responsiveness, the biological activity of Wnt/β-Catenin is context dependent, with factors such as Endothelin, BMP, or FGF2 influencing signaling output (Dunn et al., 2000; Jin et al., 2001; Viti et al., 2003; Israsena et al., 2004; Kleber et al., 2005; Ille et al., 2007). It remains to be shown whether changes in the microenvironment, faced by neural crest cells during their emigration from the neural tube, are responsible for the differential effects of β-Catenin signal activation on premigratory vs. migratory neural crest cells.

Wnt/β-Catenin signaling regulates melanocyte lineage decisions in migrating neural crest cells during a narrow time window

The virtual loss of several neural crest derivatives in *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ animals, concomitant with the generation of surplus melanoblasts in these mice, is consistent with the hypothesis that migratory neural crest cells are multipotent *in vivo*, and that neural crest lineage segregations in these cells can be influenced after their emigration from the neural tube. Alternatively, Wnt signaling might induce expansion of committed melanoblasts, as has been suggested before based on cell culture experiments (Dunn et al., 2000). However, constitutive activation of β-Catenin in melanoblasts using *Tyr-CreERT2*-driven gene expression neither led to cell expansion in the skin, confirming work by others (Delmas et al., 2007), nor to appearance of melanoblasts at ectopic sites. Therefore, altered proliferation of melanoblasts cannot explain the phenotype described in the present study.

Apart from dorsolaterally migrating cells, neural crest-derived cells along nerves innervating the skin have been reported to contribute to pigment cell formation (Adameyko et al., 2009). We used two different Cre-expressing mouse lines to exclude that β-Catenin activation promotes melanoblast generation from nerve cells rather than from migratory neural crest cells: In a first mouse line, *Cre* is expressed from *Dhh* promoter elements, which leads to Cre recombinase activity specifically in nerves from early stages onwards (Jaegle et al., 2003). Notably, we found no history of recombination in melanoblasts and melanocytes in these mice, and *Dhh-Cre*-driven β-Catenin activation neither promoted extra melanoblast generation nor suppression of other fates. In a second mouse line, inducible *Plp-CreERT2* expression has been used by others to investigate a transition from SCPs to melanocytes (Leone et al., 2003; Adameyko et al., 2009). In the present study we show that Cre-dependent recombination in these mice marks multiple neural crest derivatives, including neurons, glia, and melanocytes, when induced at early stages of neural crest development. Induction at later stages revealed a more restricted expression pattern of Cre, with recombination being detectable in both glia and melanocytes at all stages examined, including postnatally. Given the reported SCP-melanocyte transition observed in *Plp-CreERT2* mice, it is unclear why *Dhh-Cre*-mediated recombination does not label melanoblasts. Possibly, some melanocytic cells are marked independently of glia in *Plp-CreERT2* animals. Alternatively, *Dhh-Cre* and *Plp-CreERT2* might be expressed in distinct nerve cell subpopulations (Sommer, 2011). In any case, though, the dynamic activity of *Plp-CreERT2* during development allowed us to confirm that ectopic melanoblasts found in *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ animals are neither due to enhanced glia-to-melanoblast transition nor to expansion of melanoblasts: Surplus melanoblasts were only seen in *Plp-*

CreERT2/Ctnnb1$^{\Delta ex3}$ when Cre recombinase was induced at early stages to mark multiple neural crest derivatives, but not when recombination in these mice was restricted to nerve cells and melanocytes at stages E11.5 or later. These data demonstrate that β-Catenin activation can promote the generation of melanoblasts only during a narrow time window of neural crest development, after emigration of neural crest cells and before their association with nerves or engagement in the dorsolateral pathway.

The relatively narrow time window of β-Catenin responsiveness in migratory neural crest cells might also explain why surplus melanoblasts are found at ectopic sites within the embryo, rather than at their normal location in the skin. Normally, neural crest cells destined for a melanocytic fate localize to the MSA and then migrate dorsolaterally to populate the skin. Conceivably, β-Catenin activation in *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ embryos imposes a melanocytic fate in neural crest cells before they have reached their destination in the skin. This in turn causes also ventrally migrating neural crest cells to generate melanoblasts, largely at the expense of other derivatives. Evidence that melanocytes can arise from ventrally migrating cells has also been provided in zebrafish, where cells expressing melanocytic markers were found to migrate both dorsolaterally as well as ventrally (Camp and Lardelli, 2001). Intriguingly, in *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ mice ectopic melanoblasts and melanocytes are not only found in neural crest target structures, such as the autonomic ganglia, but also in tissues that are normally devoid of neural crest derivatives, such as the spleen, urogenital tract, and the diaphragm. Although this needs to be addressed, the extensive migration of β-Catenin-overexpressing melanocytic cells might reflect

processes also relevant for the high migratory capacity of cells present in aggressive melanoma.

Multipotency and lineage restrictions in avian and mouse neural crest cells

Earlier clonal assays in avian embryos indicated that at least some neural crest cells are multipotent *in vivo*, generating clones composed of both pigment and neural cell types (Bronner-Fraser and Fraser, 1988; Bronner-Fraser and Fraser, 1989; Frank and Sanes, 1991). A more recent study suggested, however, that neural crest cells are lineage-restricted already prior to their emigration from the neural tube: At early stages, cells migrate ventrally to form neural structures only, whereas at later stages cells migrate dorsolaterally to exclusively produce melanocytes (Krispin et al., 2010a; Krispin et al., 2010b). According to these data, multipotent neural crest cells would be rare in avian embryos, and the majority of neural crest cells would consist of discrete cell subpopulations generating single rather than multiple cell types (Krispin et al., 2010b). In contrast to avian embryos, there is no evidence for a temporal switch from ventral to dorsolateral migration in mouse embryos in that mouse neural crest cells migrate dorsolaterally already with the onset of neural crest migration (Serbedzija et al., 1990). In addition, single cell labeling in mouse embryos identified many clones composed of multiple neural crest derivatives, including neural cells and presumptive melanocytic cells in the dorsolateral pathway (Serbedzija et al., 1994). Despite these data, our findings indicate that the generation of distinct neural crest lineages in the mouse is also subject to temporal control mechanisms, as in avian embryos. Importantly, though, neural crest cell populations appear to be multipotent rather than lineage-restricted at premigratory as well as migratory stages, and their fate can be

influenced *in vivo* by modulation of cues controlling lineage choices. Whether multipotent cells are also multifated at different stages of neural crest development *in vivo* needs to be elucidated in future studies.

Acknowledgements

We thank, Dies Meijer, Linda Chin, A.McMahon, C.Lobe, and P. Soriano for providing transgenic animals and E.Turner for antibodies. This work was supported by the Swiss National Science Foundation, the National Center of Competence in Research “Neural Plasticity and Repair”, and the Vontobel Foundation.

The authors declare that they have no competing financial interests.

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FIGURE LEGENDS

Fig. 1. Neural crest cells express sensory and melanocyte markers sequentially and can be manipulated in a temporally controlled manner using the *Cre-loxP* system. (A-C), *In situ* hybridization analyses of *Neurog2* and *Mitf* expression on transverse sections. Presumptive neural crest cells next to the caudal neural tube express the sensory marker *Neurog2* at E10.5 while they are still *Mitf*- negative (A, B, arrowheads, adjacent sections). *Mitf* is expressed in migrating neural crest cells only one day later, in caudal regions at E11.5 (C, arrowhead). (D-I), *In vivo* fate mapping of neural crest cells either recombined with *Wnt1-Cre* or *Sox10-Cre* using the Cre-reporter line R26R. X-Gal whole mount staining and on sections demonstrates recombination in the dorsal neural tube (D, F, H, arrowheads) and in migratory neural crest cells (D, F, H, arrow) when using the *Wnt1-Cre* line. In contrast, if recombination is achieved using the *Sox10-Cre* mouse line, the neural tube remains completely unrecombined (E, G, I, open arrowheads) and recombination only occurs in migratory neural crest sells (I, arrow). F and G represent higher magnifications of the boxed area in D and E, respectively. NT, neural tube. Scalebars: 50 μm.

Fig 1:

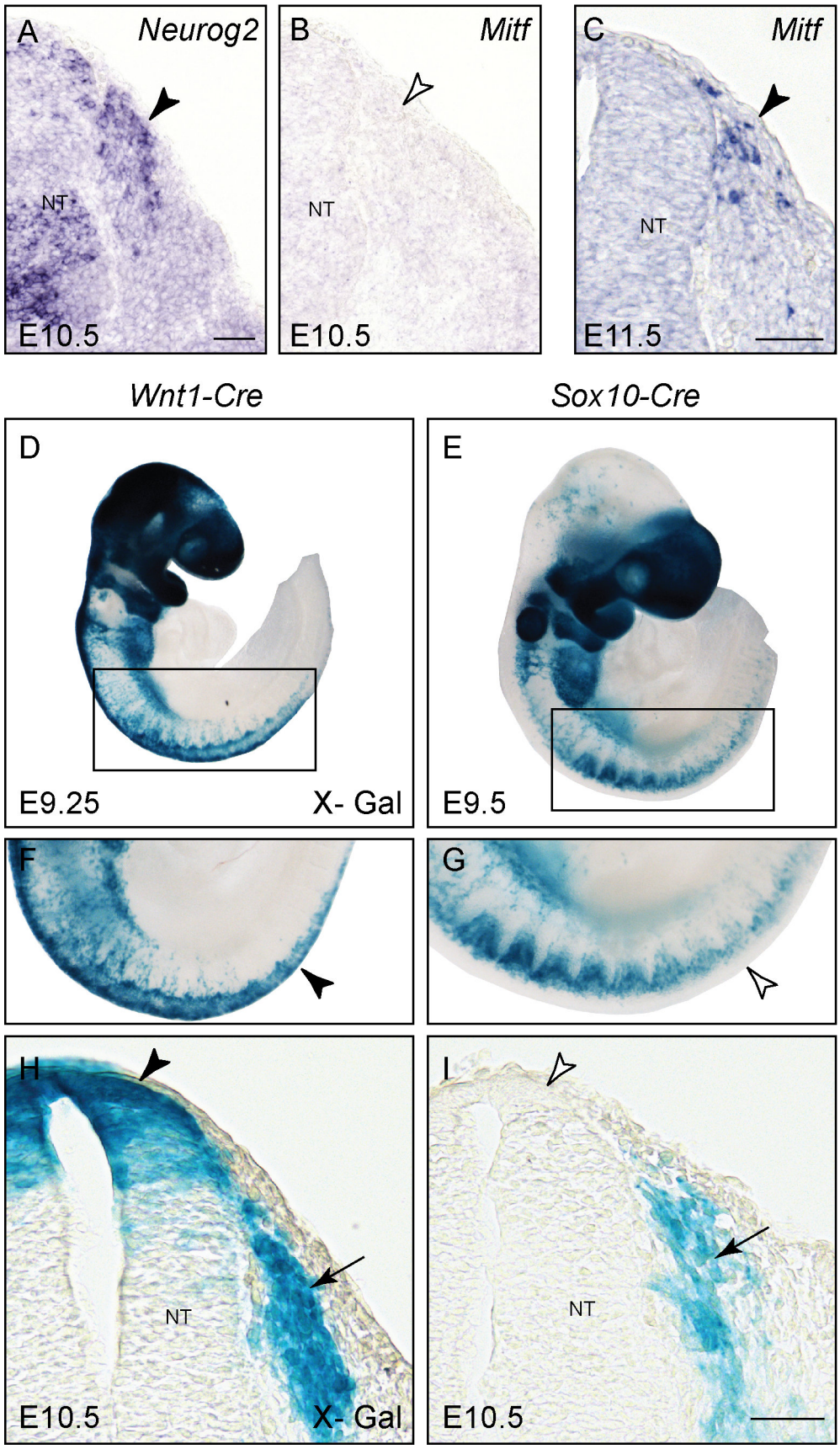

A
Neurog2
NT
E10.5
B
Mitf
NT
E10.5
C
Mitf
NT
E11.5
Wnt1-Cre
Sox10-Cre
D
E9.25
X- Gal
E
E9.5
F
G
H
NT
E10.5
X- Gal
I
NT
E10.5

Fig. 2. Formation of the melanocyte lineage is not impaired in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* embryos. *In situ* hybridization analyses on transverse sections of E11.5 and E16.5 embryos with melanoblast-specific *Mitf* and *Dct* riboprobes. (A-D), Whereas *Dct*- positive melanoblasts are readily found in the migration staging area (MSA) and around the otic vesicle (OV) of control embryos, they are absent in embryos expressing stabilized β-Catenin under the control of the *Wnt1-Cre* transgene. (E-Q), In contrast, *Mitf*- and *Dct*- positive melanoblast in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* embryos are found in normal numbers emigrating from the neural tube in caudal regions (E-H) and in the MSA and OV (I-M) at E11.5, as well as in the skin and hair follicle primordia at E16.5 (N-Q). Presence of recombined melanoblasts in hair follicle primordia is demonstrated by X-Gal staining on embryos carrying the R26R reporter (O, Q, arrows). (E, F), (G, H), (N, O) and (P, Q), respectively, are adjacent sections. (R), Quantification of *Dct*- positive melanoblasts in the MSA at E11.5 and in the skin at E16.5 reveals no significant difference in melanoblast numbers between mutant and control. Numbers of *Dct*-positive cells are calculated on transverse sections as average per MSA at E11.5 and per mm skin at E16.5, respectively. Scalebars: 50 μm.

Fig 2:

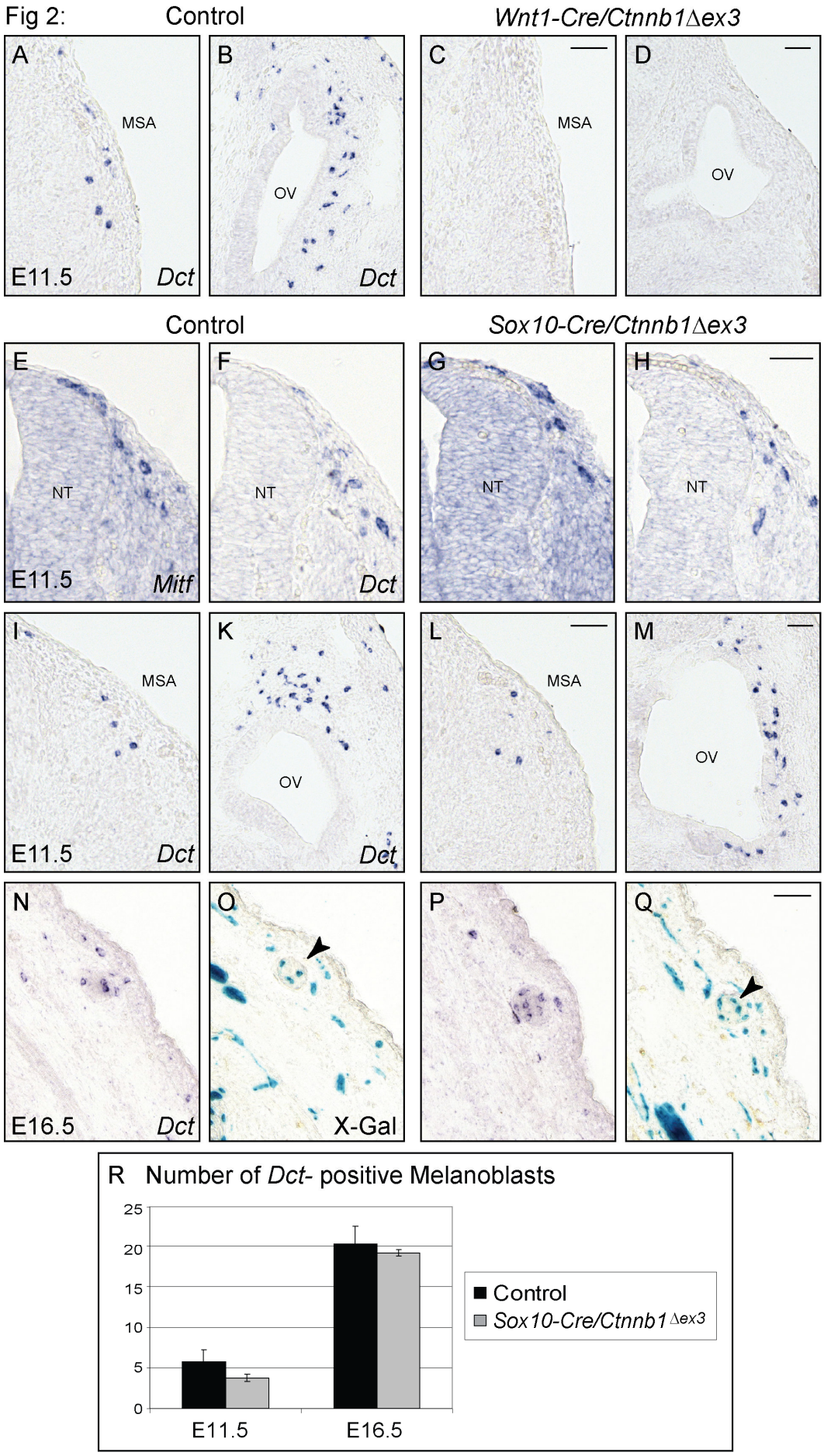

Control
Wnt1-Cre/Ctnnb1Δex3
A
MSA
E11.5
Dct
B
OV
Dct
C
MSA
D
OV
Control
Sox10-Cre/Ctnnb1Δex3
E
NT
E11.5
Mitf
F
NT
Dct
G
NT
H
NT
I
MSA
E11.5
Dct
K
OV
Dct
L
MSA
M
OV
N
E16.5
Dct
O
X-Gal
P
Q
R Number of Dct- positive Melanoblasts
25
20
15
10
5
0
E11.5
E16.5
Control
Sox10-Cre/Ctnnb1 Δex3

Fig. 3. Absence of multiple neural and non-neural derivatives of the neural crest in *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ embryos. (A-C), *In vivo* fate mapping of crest-derived craniofacial structures on whole mount heads of E14.5 embryos carrying the R26R reporter allele. Malformations of craniofacial structures in the mutant include, most prominently, lack of external ears (arrowheads) and truncated snouts frequently associated with facial clefts. (D-I), Lineage tracing of neural crest derivatives using Z/EG reporter mice visualized directly by GFP fluoresence (D, E) or with anti-GFP staining (F-I). (D, E), Whole mounts of E14.5 embryos demonstrate reduction of nerves (arrowheads and enlarged image in insets) and of the enteric nervous system (arrows). Absence of the enteric nervous system is confirmed on transverse sections at E11.5 and at E16.5 (F-I). (K-N), Immunohistochemistry on transverse sections for the autonomic neuronal marker tyrosine hydroxylase (TH) reveals a drastic reduction of autonomic neurons in the sympathetic ganglia. H, head, FL, forelimb, HL, hindlimb. Scalebars: 100 μm.

Fig 3:

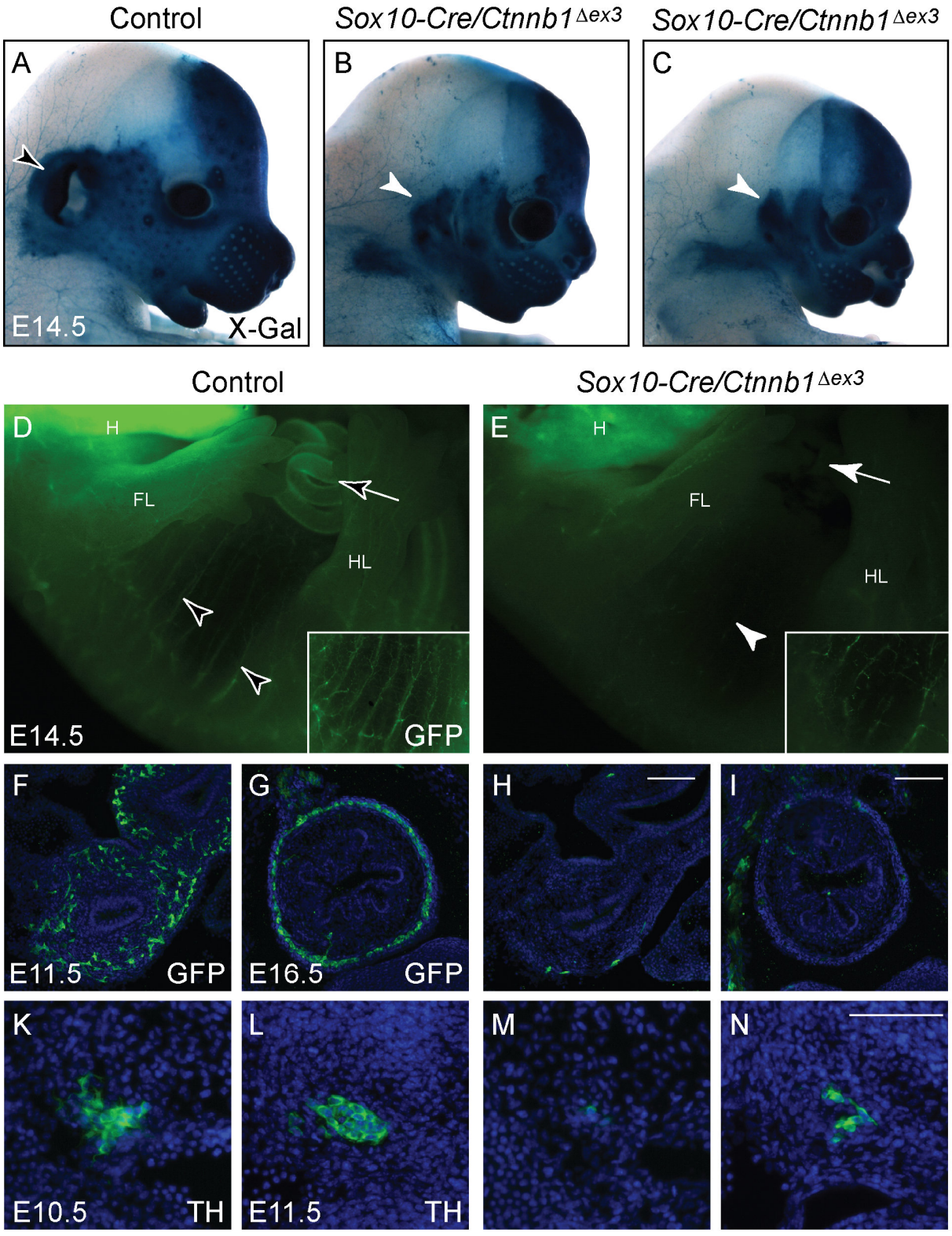

Control
Sox10-Cre/Ctnnb1Δex3
Sox10-Cre/Ctnnb1Δex3
A
B
C
E14.5
X-Gal
Control
Sox10-Cre/Ctnnb1Δex3
D
H
FL
HL
E14.5
GFP
E
H
FL
HL
F
E11.5
GFP
G
E16.5
GFP
H
I
K
E10.5
TH
L
E11.5
TH
M
N

Fig. 4. Constitutive activation of β-Catenin in migrating neural crest cells causes ectopic melanoblasts at the location of sympathetic ganglia. (A-K), *In situ* hybridization for *Mitf* and immunohistochemistry for Sox10 and Brn3a on adjacent transverse sections. Expression of stabilized β-Catenin in premigratory (*Wnt1-Cre/Ctnnb1*$^{\Delta ex3}$) as well as migratory (*Sox10-Cre/Ctnnb1*$^{\Delta ex3}$) neural crest cells leads to the formation of ectopic sensory cells, which are absent in the control (F-K). However, the number of ectopic *Mitf*-positive melanoblasts is increased in *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ embryos as compared to *Wnt1-Cre/Ctnnb1*$^{\Delta ex3}$ embryos (B-E). Additionally, enlargement of the ganglionic structure was more pronounced in *Wnt1-Cre/Ctnnb1*$^{\Delta ex3}$ embryos. Due to variations of the phenotype, examples of two different embryos for every genotype are shown. (L), Percentages of *Mitf*- and Brn3a-positive cells at the location of the sympathetic ganglia quantified on sections. The increase in *Mitf*-positive cells in *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ embryos is statistically significant (*) and is accompanied by a relative decrease in numbers of Brn3a-positive cells. *$p < 0.05$. Scalebar: 100 μm.

Fig 4:

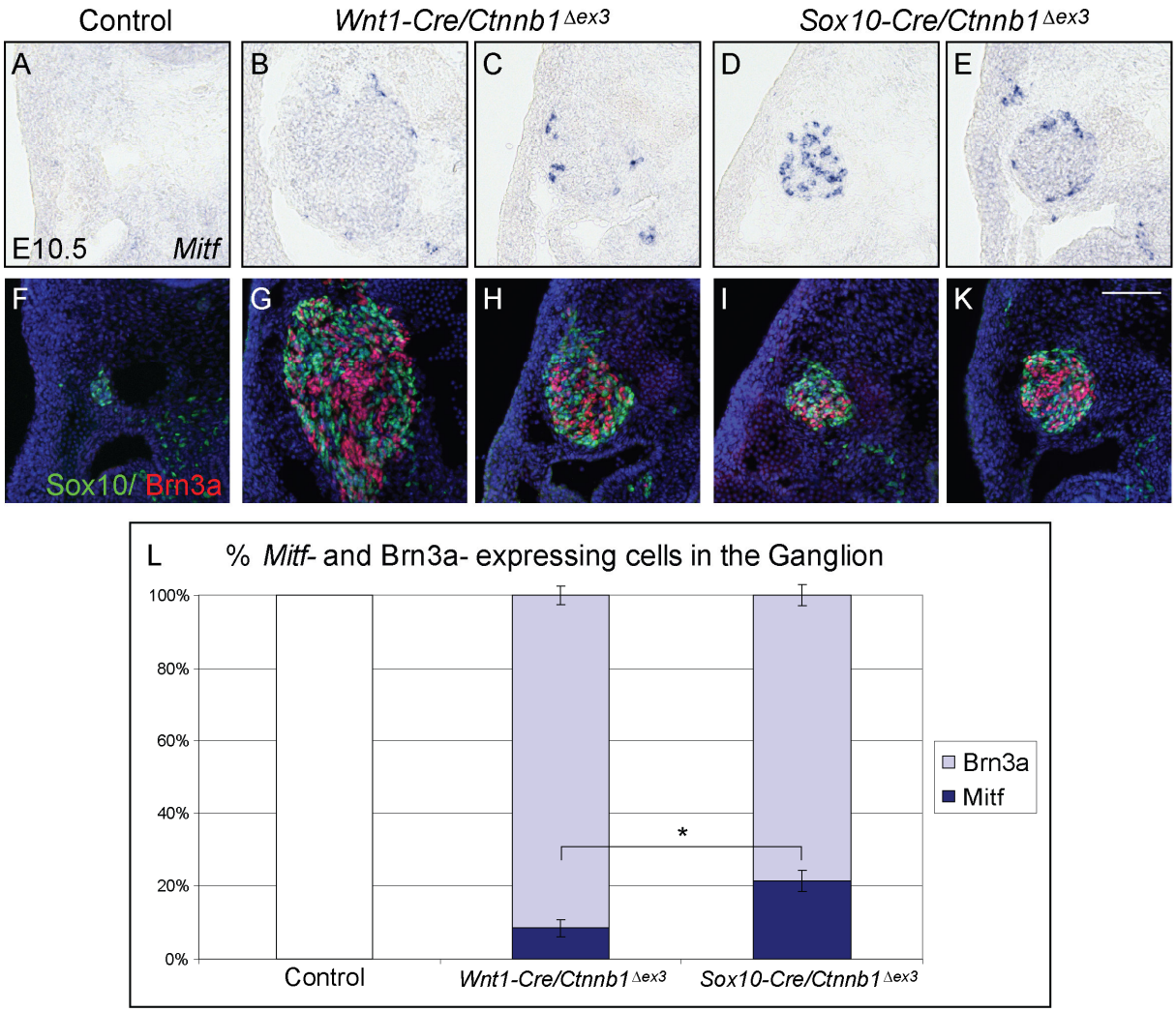

Control
Wnt1-Cre/Ctnnb1 Δex3
Sox10-Cre/Ctnnb1 Δex3
A
B
C
D
E
E10.5
Mitf
F
G
H
I
K
Sox10/ Brn3a
L % Mitf- and Brn3a- expressing cells in the Ganglion
100%
80%
60%
40%
20%
0%
*
Brn3a
Mitf
Control
Wnt1-Cre/Ctnnb1 Δex3
Sox10-Cre/Ctnnb1 Δex3

Fig. 5. Constitutive activation of β-Catenin in migrating neural crest cells causes ectopic melanoblasts at various locations throughout the embryo. Ectopic melanoblasts are detected by *in situ* hybridization experiments on transverse sections using *Mitf* (A, B) and *Dct* riboprobes (C-S). The neural crest origin of the melanoblasts is illustrated by anti-GFP or X-Gal staining using the Z/EG or R26R reporter lines, respectively. The following examples of organs populated by melanoblasts in *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ embryos are shown: tissue surrounding the sympathetic ganglia (SG) and the lung buds (LB) at E10.5 (A, B), the kidney primordium (KP, C-F) at E12.5, the diaphragm (DI, G-K) at E14.5, the spleen (SP, L-O) at E16.5, the urogenital tract: around the urethra (U) and the reproductive ducts (DU, P-S) at E16.5. Pigmentation of *Dct*-positive cells demonstrates their melanocyte identity (insets N, R). Note the absence of neural crest-derived cells in all these structures in controls. DA, dorsal aorta, , OE, oesophagus, LI, liver, S, stomach. Scalebars: 100 μm.

Fig 5:

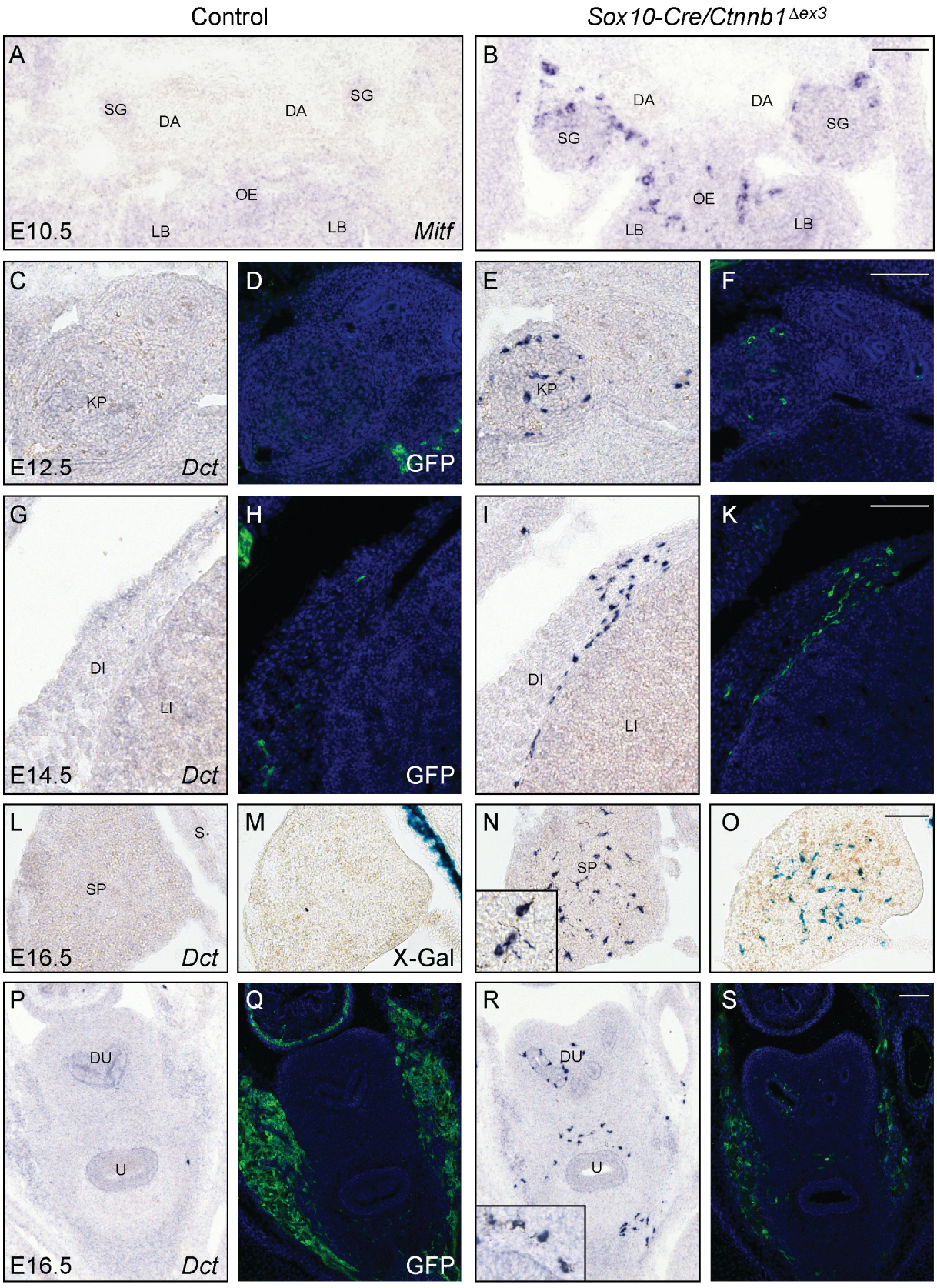
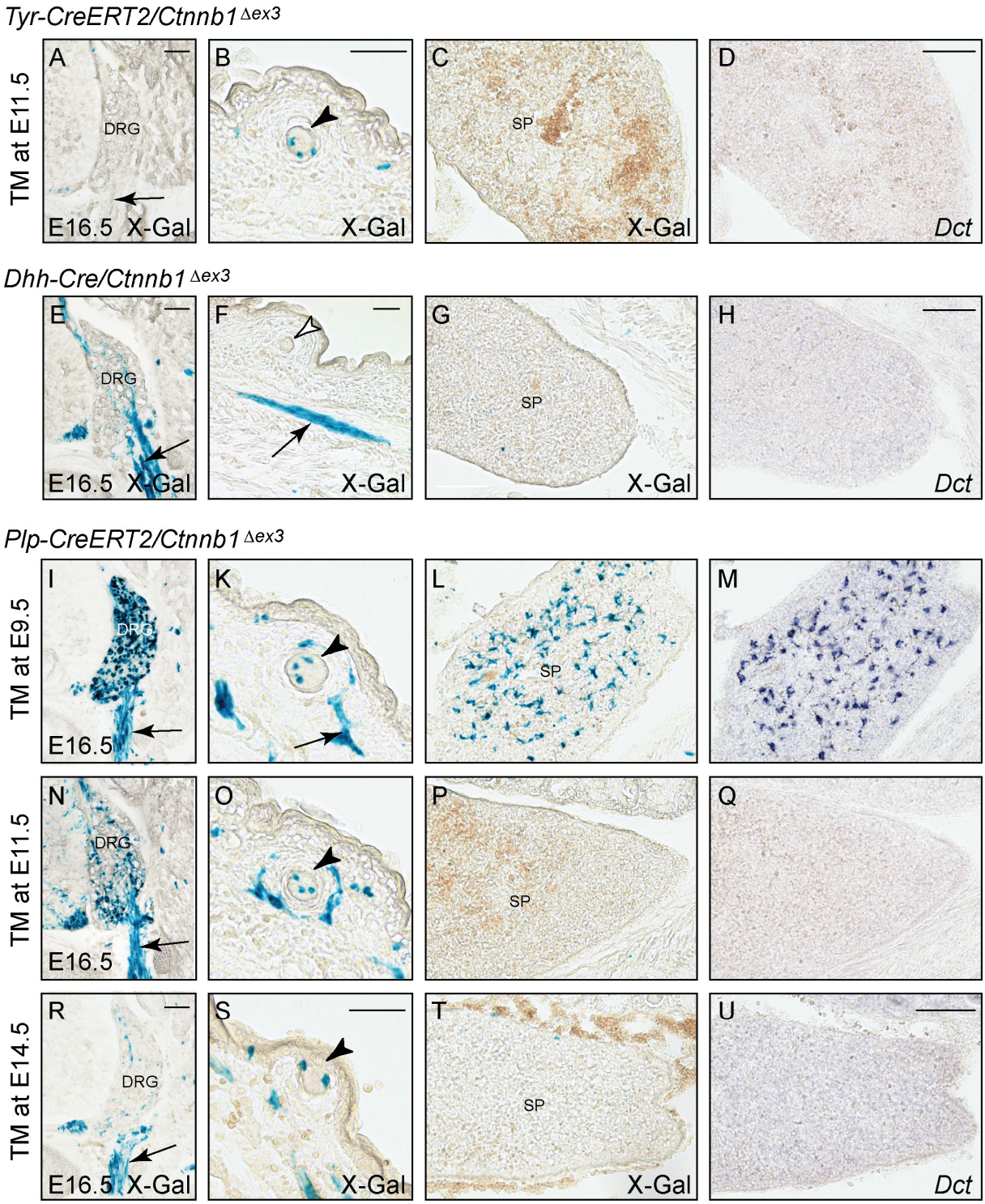

Control
Sox10-Cre/Ctnnb1Δex3
A
SG
DA
DA
SG
OE
E10.5
LB
LB
Mitf
B
DA
DA
SG
SG
OE
LB
LB
C
KP
E12.5
Dct
D
GFP
E
KP
F
G
DI
LI
E14.5
Dct
H
GFP
I
DI
LI
K
L
S
SP
E16.5
Dct
M
X-Gal
N
SP
O
P
DU
U
E16.5
Dct
Q
GFP
R
DU
U
S

Fig. 6. Ectopic melanoblasts are derived from neural crest stem cells rather than from melanoblasts or glial progenitors. X-Gal stainings on transverse sections of E16.5 embryos to identify recombined cells using R26R. *In situ* hybridization analysis on adjacent sections for *Dct* to visualize ectopic melanoblasts (last row). (A-D), Expression of stabilized β-Catenin using the *Tyr-CreERT2* mouse line does not result in formation of ectopic melanoblasts; the spleen (SP) is shown as an example (C, D). Recombined cells are detected in hair follicle primordia (B arrowhead), but not in the in the dorsal root ganglia (DRG) and nerves (A, arrow), as expected. (E-H), No ectopic melanoblasts are found in the spleen of *Dhh-Cre/Ctnnb1*$^{\Delta ex3}$ embryos (G, H). Recombination is apparent in nerves (E, F, arrows), but neither in neuronal cells of the DRG nor in melanoblasts in hair follicle primordia (E, F open arrowhead). (I-U), Expression of stabilized β-Catenin using *Plp-CreERT2* induced at different time-points. Activation of *Plp-CreERT2* by injection of Tamoxifen (TM) at E9.5 results in efficient recombination of cells in the DRG, nerves (I, K, arrows) and of melanoblasts localized to forming hair follicles (K arrowhead). Numerous ectopic melanoblasts are found in the spleen (L, M) and in other organs. When *Plp-CreERT2* is activated at E11.5 only few cells are recombined in the DRG (N), but recombination of melanoblasts still occurs (O, arrowhead). The spleen is devoid of ectopic melanoblasts (P, Q). Activation of *Plp-CreERT2* at E14.5 leads to recombination of nerve cells (R, arrow) and of melanoblasts (S, arrowhead), and to the absence of ectopic melanobasts in the spleen (T, U). Note the positive correlation between recombination of neuronal cells in the DRG and the formation of ectopic melanoblasts. Scalebars: 100 μm, except B, F, K, O, S: 50 μm.

Fig 6:


Tyr-CreERT2/Ctnnb1 Δex3
TM at E11.5
A
DRG
E16.5 X-Gal
B
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Dhh-Cre/Ctnnb1 Δex3
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5 Discussion and Outlook

5.1 Dual functions of β-Catenin in sensory lineage specification: signaling and adhesion?

We were able to demonstrate a requirement of Wnt/ β-Catenin signaling for the generation of all sensory lineages in the PNS. Neither Ngn2- dependent nor Ngn1-dependent neurogenesis occurred in the absence of β-Catenin. We speculated that specification of the Ngn2- positive lineage might depend on β-Catenin signaling functions, while the absence of Ngn1 expression possibly reflects a role of β-Catenin in mediating Cadherin-dependent cell adhesion. Recently, a very powerful tool has become available to distinguish between adhesive and signaling functions of β-Catenin. The lab of K. Basler genetically manipulated *β-catenin* in a way that signaling functions are lost but adhesive properties remain intact. Mice carrying two alleles encoding the truncated β-Catenin protein die at very early embryonic stages. To be able to discriminate between adhesive and signaling functions of β-Catenin in sensory lineage generation, we created compound mutants carrying one allele of the signaling-deficient *β-catenin* (*D164A-ΔC*) and one allele of *floxed β-catenin* (*Ctnnb1*^{flox}). To achieve recombination of the floxed allele in NCSC the *Wnt1-Cre* transgenic mouse line was used. These "β-Catenin - signaling mutants" (*Ctnnb1*$^{D164A\text{-}\Delta C\ /flox}$) are currently being analyzed in our lab. As proposed, Ngn2- positive sensory precursors were absent in these mutants. Therefore, specification of the Ngn2-dependent lineage seems to depend mainly on signaling functions of β-Catenin (preliminary results M. Gay, L. Hari and L. Sommer). Ngn1- positive cells were present, although it remains to be determined whether these cells are present in

accurate numbers. As expected from results obtained in Ngn2- deficient embryos, TrkA-, TrkB-, and TrkC- positive cells were generated at later stages. If temporal appearance of the different Trk's and their respective contribution to ganglia formation is comparable to the wildtype situation will be investigated.

To study the role of β-Catenin in sensory lineage formation at the time of DRG condensation, we plan to ablate *β-catenin* rather in migratory than in premigratory NCSC using the *Sox10-Cre* mouse line. Preliminary analysis of *Sox10-Cre Ctnnb1*$^{flox/flox}$ embryos revealed a reduction of the DRG (Fig. 9). Whether Ngn2- and/ or Ngn1- dependent lineages are present has not been analyzed yet. However, it is tempting to speculate that the smaller size of the DRG might be caused by absence of the adhesive-dependent formation of the Ngn1 lineage. If this hypothesis was true, DRGs of *Sox10-Cre Ctnnb1*$^{D164A\text{-}\Delta C\ /flox}$ mutants would be unaffected. Therefore, we will examine *Sox10-Cre Ctnnb1*$^{D164A\text{-}\Delta C\ /flox}$ animals in terms of their DRG size and of the presence for the different sensory sublineages.

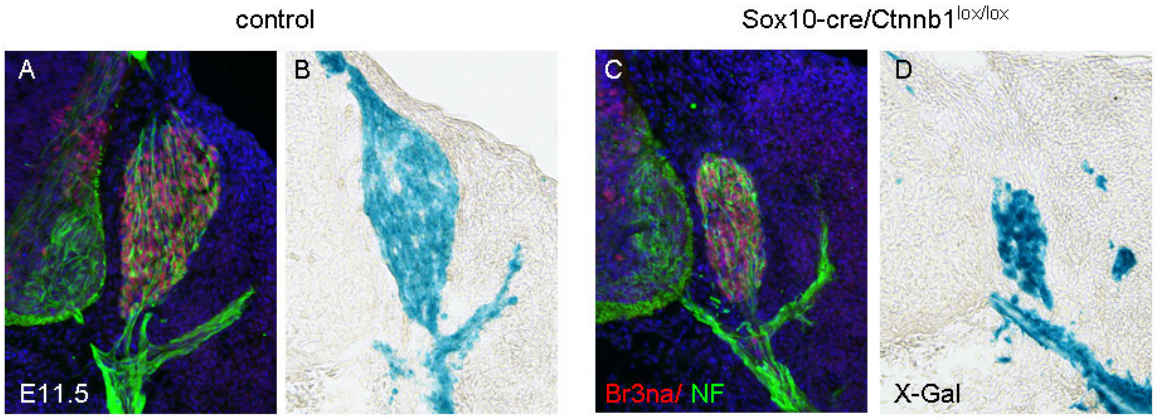

Fig. 9. Ablation of β-catenin in migratory NCSC interferes with DRG formation. Immunohistochemistry for the neuronal marker Neurofilament (NF) and the sensory marker Brn3a (A, C) and X-Gal staining on adjacent transverse sections (B, D). DRG of mutant embryos were reduced in size (C, D). unpublished data L.Hari

5.2 Mechanisms of melanocyte fate specification

Wnt as an instructive signal for melanocyte fate specification?

We successfully identified Wnt/ β-Catenin signaling as an instructive cue in sensory fate specification. In contrast, we were unable to demonstrate an instructive role for Wnt/ β-Catenin signaling in melanocyte fate determination. Although in vivo analysis of *Wnt1-Cre/Ctnnb1*$^{\Delta ex}$ and *Sox10-Cre/Ctnnb1*$^{\Delta ex}$ embryos strongly supported a role of Wnt/ β-Catenin in promoting formation of melanocytes, we encountered several difficulties while attempting to prove its instructive function. First, the time window for specification of the melanocyte lineage appears to be narrow and temporally overlapping with the window for sensory lineage commitment. Additionally, Wnt/ β-Catenin signaling seems be quite effective in promoting sensory fate acquisition. Accordingly, its function in melanocyte fate specification might be difficult do detect. Furthermore, instructiveness of a given factor can only be unambiguously demonstrated in cell culture by following the fate of a single cell in response to this factor. However, survival of single NCSC in culture is poor, making clonal cultures challenging. Culturing neural crest cells on layers of fibroblasts increases their survival, but fibroblasts might influence growth and differentiation of melanocytes (Yamaguchi et al., 2005). To analyze the instructive function of Wnt/ β-Catenin in sensory versus melanocyte fate decisions, single NSCS need to be exposed to Wnt signals at different time- points, and subsequently their fate has to be followed. Unfortunately, such an experiment is not feasible, since generation of sensory progenitors and of melanocytes requires different culture media compositions.

Given that demonstration of an instructive role for Wnt/ β-Catenin signaling in melanocyte fate specification was unsuccessful, we attempted to verify the presence

of an activated Wnt/ β-Catenin pathway in melanoblasts. The β-Catenin- dependent nuclear response to Wnt can be visualized using BAT GAL transgenic mice, which express the *lacZ* gene under the control of β-Catenin-TCF -responsive elements (Maretto et al., 2003). Unexpectedly, we found no correlation between Mitf- positive melanoblasts and expression of β-Galactosidase in vivo. However, such a correlation was also not detectable between Ngn2- positive sensory precursors and BAT GAL expression. Thus, the BAT-GAL mouse line is not a suitable tool to detect the presence of an activated Wnt/ β-Catenin pathway in melanoblasts.

Temporal control of NCSC in response to Wnt signals

Our data are consistent with the hypothesis of a temporal control of Wnt signaling in neural crest lineage generation. In such a model, Wnt/ β-Catenin signaling would promote sensory fate in early or premigratory NCSC while in migrating NCSC it would regulate melanocyte fate decision (Fig. 10).

To provide evidence for a stage-dependent requirement of Wnt/ β-Catenin in sensory and melanocyte lineage generations, Wnt signaling has to be abolished in NCSC at different time- points. Ablation of β-Catenin using different *Cre*-lines has turned out to be an inadequate tool, however, since loss of protein lagged behind conditional gene deletion. Possibly, Wnt signaling in neural crest stem cells can be blocked using small chemical compounds. Several molecules have been recently identified to inhibit the intracellular Wnt signaling pathway (Huang et al., 2009; Thorne et al., 2010; Park et al., 2005). Application of these Wnt inhibitors has not been established in neural crest cultures yet. Inhibition of Wnt signaling at early time- points supposedly impairs generation of sensory and melanocyte lineages, while at later time-points

suppression of the Wnt pathway might only interfere with formation of melanocytes. However, due to the narrow and temporally overlapping time windows for specification of sensory and melanocyte lineages, it is questionable whether such an experiment provided the expected results.

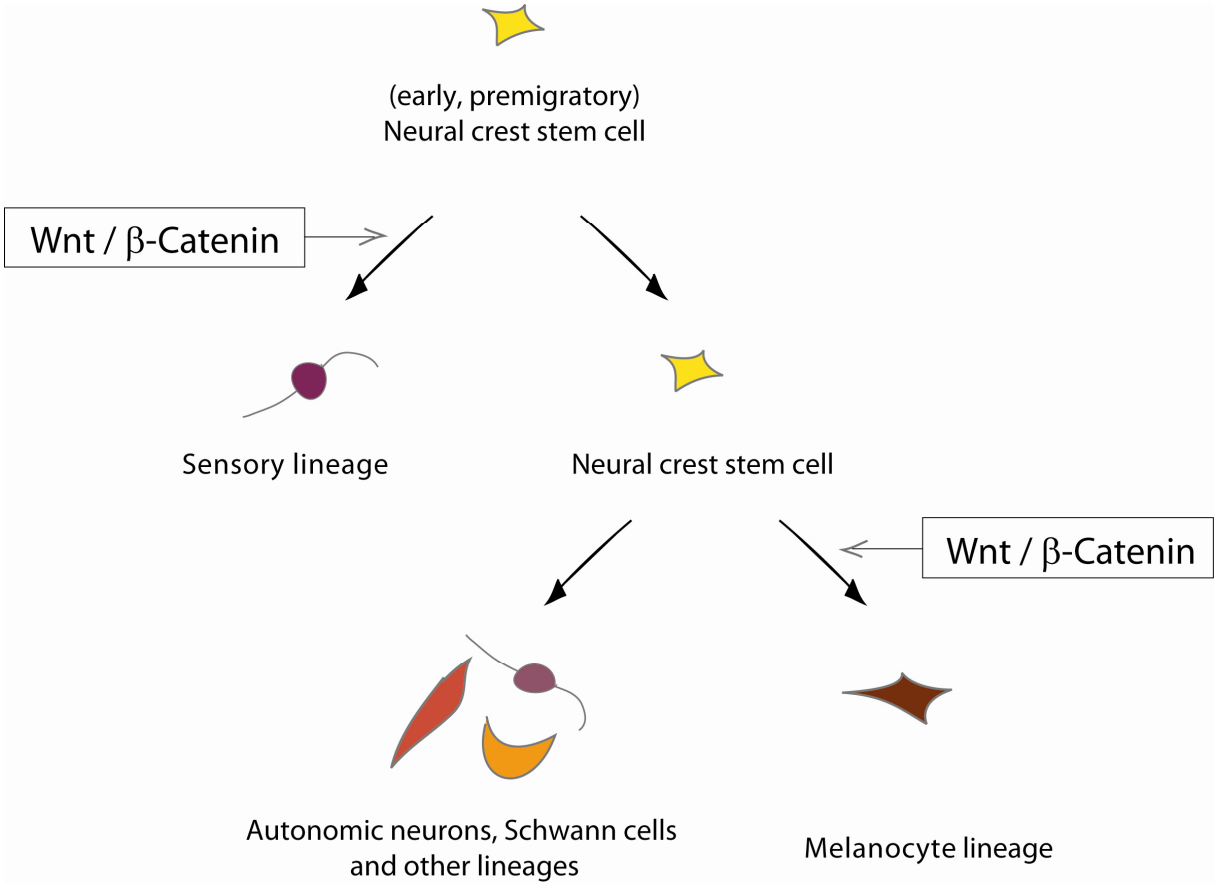

Fig. 10. Model of temporal control of neural crest lineage generation by Wnt/ β-catenin signaling. In premigratory NCSCS, Wnt/ β-Catenin would control sensory lineage decisions while in migrating NCSC it would promote formation of melanocytes. Adapted from Sommer, 2011.

Interplay between Wnt and other signaling pathways

Specificity for reiterated Wnt signals can be achieved through establishment of cell-intrinsic differences, which consequently result in different responses to external stimuli. Cell- extrinsic signals can change the intrinsic state of a cell by influencing it

in sequential order and thereby, change the cell's response to the next signal. Alternatively, one signal can modulate the response to another signal, or more than one signal is needed simultaneously to activate specific target genes (Raible and Ragland, 2005). Wnt signaling pathways have been shown to interact with multiple other signaling cascades. Some examples of how Wnt and other signaling pathways are integrated in regulating cell lineage decisions and organ patterning during development are described in the following paragraph. Whether such relations also occur in specification of sensory and melanocyte lineages has to be to carefully analyzed.

Wnt and BMP for instance have been shown to cooperate in dorsoventral patterning of xenopus mesoderm, in promotion of osteoblast differentiation from bone mesenchymal stem cells, or in lineage decisions between neural and epidermal fate in the chick epiblast (Hoppler and Moon 1998; Lin and Hankenson, 2011; Wilson et al., 2001). A balance of Wnt, BMP and FGF signals regulates the choice of mammalian head mesenchymal stem cells to enter the chondrogenic lineage (Maruyama et al., 2010), and Wnt and FGF signaling cooperate in patterning of the anteroposterior axis in xenopus neural ectoderm (McGrew et al., 1997). Wnt and Notch signaling interactions were first discovered in the context of the development and patterning of the drosophila wing (Hayward et al., 2008). Notch was shown to interact with the Wnt signaling pathway not only on a transcriptional level, but also to be able to physically associate with unphosphorylated β-Catenin (Kwon et al., 2011). Interactions between Wnt and TGF-β signaling pathways have been demonstrated in midbrain development, where TGF-β signal activation counteracts Wnt-induced proliferation of neuroepithelial cells (Falk et al., 2008). TGF-β and canonical Wnt signaling were also shown to cooperate in eye development (Grocott et al., 2011).

Physical association between downstream targets of the two pathways, β-Catenin, LEF/ TCF and Smad proteins, have been detected in various situations (Nishita et al., 2000; Labbe et al., 2000; Edlund et al., 2004). Antagonistic effects for sonic hedgehog (SHH) and canonical Wnt signaling were demonstrated to be involved in dorso- ventral patterning of the vertebrate neural tube (Ulloa and Marti, 2010).

Factors potentially involved in melanocyte fate specification in addition to Wnt

Wnt/ β-Catenin signaling alone might not be sufficient to induce melanocytic fate. Both, SCF and EDN growth factors were shown to be required for survival, proliferation, differentiation, and migration of melanoblasts, but not for their initial generation from neural crest (Saldana-Caboverde and Kos, 2010; Sommer, 2011). Accordingly, Wnt3a together with SCF and EDN was used to efficiently generate melanocytes from human embryonic stem cells (hESC). Melanocytes were only found in cultures supplemented with Wnt3a and EDN, or with all three growth factors. Neither of the factors alone was able to promote the formation of melanocytes (Fang et al., 2006).

In quail neural crest cultures, BMP-4 was shown to inhibit specification of melanoblasts and instead promote neural fates (Jin et al., 2001). However, in *Wnt1-Cre*/*Smad4*$^{flox/flox}$ embryos, where BMP signaling is depleted in neural crest cells, formation of melanoblasts appeared to be normal (unpublished observation, L. Hari and L. Sommer).

To current knowledge, only the combined absence of Wnt1 and Wnt3a results in lack of melanocytes, similar to loss of β-Catenin in *Wnt1-Cre*/*Ctnnb1*$^{flox/flox}$ embryos (Ikeya

et al., 1997). However, several other Wnt ligands are expressed in the dorsal neural tube and in surrounding regions around the time of neural crest migration (Summerhurst et al., 2008; http://www.tcd.ie/Zoology/research/WntPathway/wnt.php). For most of these Wnts, a potential role in melanocyte development has not been investigated. Some Wnts might have redundant functions, others possibly interfere with each other in modulating cellular responses. Non-canonical Wnt5 for instance is known to inhibit canonical Wnt signaling (Topol et al., 2003). Based on expression patterns, non- canonical Wnt4 is a conceivable candidate to be involved in melanocyte development, since it is expressed in the dorsal neural tube with increasing intensity from E9.5 to E11.5. Wnt7a, Wnt7b, and Wnt8 seem to be expressed in the neural tube, while Wnt6, Wnt11 and Wnt16 expression can be detected next to the neural tube in regions where melanoblasts are located in the MSA. The exact expression patterns of different Wnt ligands and their frizzled receptors as well as a potential role in melanocyte development have to be further evaluated.

Secreted Wnt inhibitors like Dickkopf (DKK) or secreted frizzled related proteins (sFRPs) might also be involved in regulating Wnt- dependent fate decisions of NCSC in a temporally controlled manner. In the skin of human palms, Dkk1 secreted from fibroblasts was shown to inhibit melanocyte proliferation and to suppress expression of melanosomal proteins. This effect of Dkk1 was mediated by decreasing intracellular levels of β-Catenin and subsequent downregulation of *mitf* (Yamaguchi et al., 2004). sFRP2 expression was detected in the neural tube at E9.5 and E10.5, while its expression is downregulated around E11.5, the time when first Mitf- positive melanoblasts appear (Lescher et al., 1998). sFRP1 and sFRP2 single and double

knock out mice are available, but formation of melanoblasts has unfortunately not been examined in these mutants (Satoh et al., 2003).

5.3 Generation of melanocytes from glial precursors

The idea of schwann cell precursors (SCPs) detaching from nerves and giving rise to melanoblasts is fascinating and has been shown to occur in avian development (Adameyko et al., 2009). Furthermore, evidence for the existence of a bipotent glial-melanocyte precursor has been provided in avian culture systems (Dupin and Le Douarin, 2003). Such a bipotent precursor can be expanded upon administration of EDN3, and EDN3 is able to reverse differentiation of both melanocytes and glial cells back to the bipotent precursor state (Dupin et al., 2000; Dupin et al., 2003). In mouse embryos, generation of melanoblasts from SCP has not been conclusively verified. However, upon injury of mouse adult sciatic nerves, cells assumed to be of glial origin give rise to pigmented melanocytes (Rizvi et al., 2002). This points to a close relationship between glial and melanocyte lineages also in mammalians.

Evidence of a transition from SCPs to melanoblasts in the mouse was provided using in vivo lineage tracing experiments with *Plp-CreERT2* (Adameyko et al., 2009). However, several transgenic *Cre* mouse lines do not recombine in neural crest lineages as expected from the expression patterns of the corresponding Cre- driving promotors. We observed expression of *Plp-CreERT2*, *P0-Cre, Tyr-CreERT2, Dct-Cre*, and *Dct-LacZ*, in neural crest progenitors instead of either in the glial or in the melanocyte lineage, respectively. Moreover, the time- window for SCP to melanocyte transition was claimed to be narrow and only to be active early, before expression of later glia- specific Cre lines starts. Accordingly, *Dhh-Cre* labelled SCP did not give

rise to melanocytes (Adameyko et al., 2009). Further Cre- lines might be worth considering as tools to examine a potential glial to melanocyte transition. The *P0A-Cre* line exhibits a promising expression pattern not in NCSC but early in nerves (Zheng et al., 2008; Giovannini et al., 2000). I suggest investigating the existence of recombined melanocytes in *P0A-Cre/ ROSA26* embryos. Krox20-Cre, BFABP-Cre, S100b-Cre, and GFAP-CreERT2 lines are also available (Zhu et al., 2002; Hegedus et al., 2007; Tanaka et al., 2008; Chow et al., 2008), but predicted expression starts too late in the glial lineage to show the presumptive transition towards melanocyte fate.

5.4 Migration of ectopic melanoblasts in *Sox10-Cre/Ctnnb1$^{\Delta ex}$* embryos

The mechanisms involved in the changed migratory behaviour of melanoblasts expressing a stabilized form of β-Catenin might enable us to gain insight into the mechanisms of neural crest and melanoblast migration in general. Moreover, some of the processes involved in this extensive migration might reflect processes also relevant for the high migratory capacity of cells present in aggressive melanoma. Furthermore, our results suggest that specification and migration of neural crest cells are to some extent independently regulated.

Neural crest cells of *Sox10-Cre/Ctnnb1$^{\Delta ex}$* embryos embarking on the ventral pathway are specified to adopt melanocyte fate in response to stabilized β-Catenin but are still excluded from the dorsolateral pathway. Once the neural crest cells reach the location of the sympathetic ganglia they normally upregulate N-Cadherin and

condensate into a ganglionic structure (Kasemeier-Kulesa et al., 2006). However, at least at later developmental stages, the ectopic β-Catenin- overexpressing melanocytes do not express N-Cadherin (L. Hari and L. Sommer, unpublished). Therefore they might be unable to condensate into sympathetic ganglia and continue to migrate. In support of this hypothesis ectopic Brn3a- positive precursors stall in the location of the sympathetic ganglia and form a ganglionic structure, as they normally do in the DRG. Whether Brn3a- positive ectopic cells express N-Cadherin has to be analyzed.

What cues might guide the ectopic melanoblasts along distinct pathways once they have left the area of the sympathetic ganglia, and what mechanisms prompt them to settle down in certain locations? In the following paragraphs I will suggest some molecules potentially involved in these processes. I recommend carefully analyzing their expression patterns in control neural crest cells, in control melanoblasts, and in ectopic melanoblasts. Presence of the corresponding ligands has to be determined also in the surrounding tissues.

Plausible candidates are, of course, surface receptors and their ligands regulating neural crest migration into the dorsolateral pathway, such as neuropilin – semaphoring and c-Kit. Surprisingly, c-Kit is expressed in ectopic melanoblasts (L. Hari and L. Sommer, unpublished). Expression of the corresponding growth factor SCF in the dermomyotome is probably too far away to attract the cells and lead them to migrate into the skin. Alternatively, SCF might not be expressed in the skin at later developmental stages.

CXCL12, formerly known as stromal cell-derived-factor1 (SDF-1) is one of the few secreted proteins known to attract neural crest cells. CXCL12 is expressed in the

sclerotome, adjacent to the future SGs and in regions surrounding the gut. Migrating neural crest cells express the corresponding receptor CXCR4 (Gammill and Roffers-Agarwal, 2010; Kasemeier-Kulesa et al., 2010). Overexpression of CXCR4 in non-SG-destined neural crest cells induces them to migrate aberrantly toward the SGs, suggesting a role of CXCR4- CXCL12 interactions in attracting ectopic melanoblasts to the sympathetic ganglia rather than guiding their migration beyond (Kasemeier-Kulesa et al., 2010). On the other hand, CXCR4- CXCL12 was shown to regulate chemotactic migration of tumour cells to secondary organs and to be associated with metastatic spread of melanoma (Gassmann et al., 2009). In zebrafish the Wnt/ β-catenin pathway, in combination with FGF, was shown to regulate differential expression of the chemokine receptors cxcr4b and cxcr7b (Aman and Piotrowski, 2008). Whether CXCR4 is expressed in orthothopic or ectopic melanoblasts and what role it might play in guiding ectopic melanoblasts has to be investigated.

Originally discovered as an oncogene, the c-Met receptor tyrosine kinase and its ligand Hepatocyte growth factor (HGF) are thought to play an important role in both melanocyte development and melanoma metastasis. c-Met was shown to be a direct transcriptional target of Mitf (McGill et al., 2006). Furthermore, in metallothionein promoter-driven HGF transgenic mice ectopic melanocytes were found to be situated aberrantly within regions of abnormal HGF expression (Takayama et al., 1996). HGF is normally derived from keratinocytes and has mitogenic and melanogenic effects on melanoblasts (Hirobe, 2011).

The cell adhesion molecule MCAM has been associated with melanoma progression and metastatic potential. Up-regulation of MCAM, together with loss of E-Cadherin, is one of the crucial events that allow melanoma cells to invade the dermis and to

progress to the vertical growth phase. MCAM was demonstrated to be a downstream target of Pax3 (Medic et al., 2011). MCAM is also a target of Notch transactivation, Notch1 was identified to be a transforming oncogene in human melanocytes, possibly by upregulation of MCAM (Pinnix et al., 2009). It will be interesting to analyze whether the ectopic melanoblasts in *Sox10-Cre/Ctnnb1*$^{\Delta ex}$ embryos express MCAM. The ectopic melanoblasts lack expression of E-Cadherin (L. Hari and L. Sommer, unpublished), but also melanoblasts migrating through the dermis before localizing to hair follicles are E-Cadherin negative.

Another cell surface molecule known to promote cell motility is melanoma chondroitin sulfate proteoglycan (MCSP), also called chondroitin sulphate proteoglycan 4 (CSPG4). MCSP was shown to be a direct target of PAX3, and was implicated to be a mediator of malignant potential in melanoma (Medic et al., 2011). Normal melanocytes express little or no MCSP, whereas elevated levels of MCSP are detected in melanoma cells. MCSP can increase cell motility by modifying the organization of the cytoskeleton through modulating the activity of Rho family GTPases. Moreover, it acts as a coreceptor for integrins regulating cell adhesion properties to the extracellular matrix (ECM) (Yang et al., 2009).

Small Rho-GTPase- dependent migration of neural crest cells has been associated with the non-canonical Wnt PCP pathway (DeCalisto et al., 2005; Clay and Halloran, 2011). However, neural crest- specific deletion of the small Rho-GTPases Cdc42 or Rac1 performed in our lab revealed that Cdc42 and Rac1 are dispensable for neural crest migration (Fuchs et al., 2009). Rather Cdc42 and Rac1 were required for proliferation and self- renewal of neural crest progenitors in postmigratory target structures. Unfortunately, the formation of melanoblasts in these mouse mutants has

not been addressed. Irrespective of a possible function of Rac1 and Cdc42 in melanocyte development, small Rho-GTPases might be involved in aberrant migration of β- Catenin overexpressing melanoblasts. It remains to be analysied whether Rac1, Cdc42, and other small Rho- GTPases are activated in ectopic melanoblasts of *Sox10-Cre/Ctnnb1*$^{\Delta ex}$ embryos. Depending on the results, it would be interesting to assess the formation of ectopic melanoblasts due to β- Catenin stabilization in a Rac1- or Cdc42- deficient background.

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7 Publications

1. **Hari, L.**, Miescher, I., Shakhova, O., Suter, U., Taketo, M., Richardson, W.D., Kessaris, N., and Sommer L. (2011). Temporal control of neural crest lineage generation by Wnt/ b-Catenin signaling. Development, current status in revision.

2. Lee, H.Y., Kleber, M., **Hari, L.**, Brault, V., Suter, U., Taketo, M.M., Kemler, R., and Sommer, L. (2004). Instructive role of wnt/ beta-catenin in sensory fate specification in neural crest stem cells. Science *303*, 1020-1023.

3. **Hari, L.**, Brault, V., Kleber, M., Lee, H.Y., Ille, F., Leimeroth, R., Paratore, C., Suter, U., Kemler, R., and Sommer, L. (2002). Lineage-specific requirements of beta-catenin in neural crest development. J Cell Biol *159*, 867-880.

4. Paratore, C., Hagedorn, L., Floris, J., **Hari, L.**, Kleber, M., Suter, U., and Sommer, L. (2002). Cell-intrinsic and cell-extrinsic cues regulating lineage decisions in multipotent neural crest-derived progenitor cells. Int J Dev Biol *46*, 193-200.

8 Curriculum vitae

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Education

Since 02/2009	**PhD in Cell Biology, University of Zurich, Switzerland** Prof. Dr. L. Sommer, Institute of Anatomy: "Role of Wnt/ β-Catenin Signaling in Neural Crest Development"
07/2002 - 02/2009	**Research Assistant, ETH Zurich and University of Zurich, Switzerland** Prof. Dr. L. Sommer, Institute of Cell Biology, ETH Zurich and Institute of Anatomy, University of Zurich
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9 Acknowledgements

I am extremely happy to finally be able to complete my PhD studies. For an extended period of time, it was uncertain whether this would ever be possible. I would not have succeeded without the help and support of many people:

First, I express my gratitude to Prof. Dr. Lukas Sommer who gave me the possibility to fight my way back to work, step by step. I am very thankful for his support and for the fact that he never doubted an instance I did my best. I highly enjoyed our innumerable inspiring scientific discussions and the enthusiasm we shared. I very much appreciated the way he allowed us to perform research to our personal responsibility. It was a pleasure to perform my PhD in his lab.

I thank Prof. Dr. Esther Stöckli and Prof. Dr. Konrad Basler for their participation in my thesis committee and for the very helpful discussions on our meetings.

I thank Prof. Dr. Ueli Suter for his support and encouragement during my time at the Institute of Cell Biology.

I am very grateful for the assistance of Prof. Dr. Michael Hengartner who in his open-mindedness enabled me to finish my PhD at the University of Zurich.

I thank present and former members of the Sommer- Group for their help, interesting discussions and the pleasant times we had. I would like to especially thank Iris, Olga, Nessy, Stine, Christine, Sebastian, David, Fabian, Heiko, Sven, Kalina and Max. I also got support from members of the Suter and Loffing Group and from other colleagues at the IZB and at the Institute of Anatomy, including technical and administrative staff.

I thank my medical doctor Silvio Schaller for his continuous assistance and support.

Most important was the love and support of Christian, of my family and of my friends. Thank you!